

# **APPENDIX A**

**(CLEAN VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS)**

**(Serial No. 10/002,750)**



PATENT  
Attorney Docket 5148US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL 750547536 US

Date of Deposit with USPS: November 15, 2001

Person making Deposit: Lynette Eliason

APPLICATION FOR LETTERS PATENT

for

COMPLEMENTING CELL LINES

Inventors:  
Ronald Vogels  
Menzo Havenga  
Majid Mehtali

Attorney:  
Allen C. Turner  
Registration No. 38,581  
TRASK BRITT  
P.O. Box 2550  
Salt Lake City, Utah 84110  
(801) 532-1922

## TITLE OF THE INVENTION

### COMPLEMENTING CELL LINES

#### CROSS-REFERENCE TO RELATED APPLICATION

**[0001]** This application is a continuation-in-part of application Serial No. 09/713,678, filed November 15, 2000, pending.

#### TECHNICAL FIELD

**[0002]** The invention relates to the field of biotechnology generally and, more specifically, to adenoviral-based complementing cell lines.

#### BACKGROUND OF THE INVENTION

**[0003]** Typically, vector and packaging cells have to be adapted to one another so that they have all the necessary elements, but they do not have overlapping elements that lead to replication-competent virus by recombination. Therefore, the sequences necessary for proper transcription of the packaging construct may be heterologous regulatory sequences derived from, for example, other human adenovirus (Ad) serotypes, nonhuman adenoviruses, other viruses like, but not limited to, SV40, hepatitis B virus (HBV), Rous Sarcoma Virus (RSV), cytomegalovirus (CMV), etc. or from higher eukaryotes such as mammals. In general, these sequences include a promoter, enhancer and poly-adenylation sequences.

**[0004]** PER.C6 (ECACC deposit number 96022940) is an example of a cell line devoid of sequence overlap between the packaging construct and the adenoviral vector (Fallaux *et al.*, 1998). Recombinant viruses based on subgroup C adenoviruses, such as Ad5 and Ad2, can be propagated efficiently on these packaging cells. Generation and propagation of adenoviruses from other serotypes, like subgroup B viruses, has proven to be more difficult on PER.C6 cells. However, as described in European patent application 00201738.2, recombinant viruses based on subgroup B virus Ad35 can be made by co-transfection of an expression construct containing the Ad35 early region-1 sequences (Ad35-E1). Furthermore, Ad35-based viruses that are deleted for E1A sequences were shown to replicate efficiently on PER.C6 cells. Thus, the E1A proteins of

Ad5 complement Ad35-E1A functions, whereas, at least part of the E1B functions of Ad35 are necessary. This serotype specificity in E1B functions was recently also described for Ad7 recombinant viruses. In an attempt to generate recombinant adenoviruses derived from subgroup B virus Ad7, Abrahamsen *et al.* (1997) were not able to generate E1-deleted viruses on 293 cells without contamination of wild-type (wt) Ad7. Viruses that were picked after plaque purification on 293-ORF6 cells (Brough *et al.*, 1996) were shown to have incorporated Ad7-E1B sequences by nonhomologous recombination. Thus, efficient propagation of Ad7 recombinant viruses proved possible only in the presence of Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B proteins are known to interact with cellular, as well as viral, proteins (Bridge *et al.*, 1993; White, 1995). Possibly, the complex formed between the E1B-55K protein and E4-ORF6 which is necessary to increase mRNA export of viral proteins and to inhibit export of most cellular mRNAs, is critical and in some way serotype-specific.

#### DESCRIPTION OF THE INVENTION

[0005] The present invention provides new packaging cell lines capable of complementing recombinant adenoviruses based on serotypes other than subgroup C viruses, such as serotypes from subgroup B like adenovirus type 35.

[0006] In one aspect, the invention provides packaging cell lines capable of complementing recombinant adenovirus based on a serotype of subgroup B, preferably of serotype 35. With the terms "based on or derived from an adenovirus" is meant that it utilizes nucleic acid corresponding to nucleic acid found in the serotype. The utilized nucleic acid may be derived by PCR cloning or other methods known in the art.

[0007] In one aspect, the new packaging cells are derived from primary, diploid human cells such as, but not limited to, primary human retinoblasts, primary human embryonic kidney cells or primary human amniocytes. Transfection of primary cells or derivatives thereof with the adenovirus E1A gene alone can induce unlimited proliferation (immortalization), but does not result in complete transformation. However, expression of E1A in most cases results in induction of programmed cell death (apoptosis), and occasionally immortalization is obtained (Jochemsen *et al.*, 1987). Co-expression of the E1B gene is required to prevent induction of apoptosis and for complete

morphological transformation to occur (reviewed in White, 1995). Therefore, in one aspect of the invention, primary human cells or derivatives thereof are transformed by expression of adenovirus E1 proteins of a subgroup other than subgroup C, preferably subgroup B, more preferably adenovirus type 35. The combined activity of the E1A and E1B proteins establishes indefinite growth of the cells and enables complementation of recombinant adenoviruses.

[0008] The complete morphological transformation of primary cells by adenovirus E1 genes is the result of the combined activities of the proteins encoded by the E1A and E1B regions. The roles of the different E1 proteins in lytic infection and in transformation have been studied extensively (reviewed in Zantema and van der Eb, 1995; White, 1995, 1996). The adenovirus E1A proteins are essential for transformation of primary cells. The E1A proteins exert this effect through direct interaction with a number of cellular proteins that are involved in regulation of transcription. These include the pRB family of proteins, p300/CBP and TATA binding protein. In addition to this E1A increases the level of p53 protein in the cells. In the absence of adenovirus E1B activity the rise in p53 levels leads to the induction of apoptosis. Both proteins encoded by the E1B region counteract the induction of apoptosis although by different mechanisms. E1B-21K seems to counteract apoptosis in a manner similar to Bcl-2 via interaction with the effector proteins downstream in the apoptosis pathway (Han *et al.*, 1996), whereas E1B-55K functions through direct interaction with p53. Importantly, the molecular mechanism by which the E1B-55K proteins of Ad2 and 5 (subgroup C) and Ad12 (subgroup A) function in the ability to neutralise p53 may differ. Whereas Ad5 E1B-55K binds p53 strongly and the complex localises to the cytoplasm, Ad12 E1B-55K binds p53 weakly and both proteins are localised in the nucleus (Zantema *et al.*, 1985; Grand *et al.*, 1999). Both proteins, however, inhibit the transactivation of other genes by p53 (Yew and Berk, 1992).

[0009] In rodent cells, the activity of E1A together with either E1B-21K or 55K is sufficient for full transformation although expression of both E1B proteins together is twice as efficient (Rao *et al.*, 1992). In human cells however, the activity of the E1B-55K protein seems to be more important given the observation that E1B-55K is indispensable for the establishment of transformed cells (Gallimore, 1986).

[0010] Example 6 hereof describes the generation of pIG270. In this construct the Ad35-E1 genes are expressed from the hPGK promoter and transcription is terminated by the HBVpA. The hPGK promoter constitutes a HincII-EcoRI fragment of the promoter sequence described by Singer-Sam *et al.* (1984). The HBVpA is located in a BamHI-BglII fragment of the Hepatitis B virus genome (Simonsen and Levinson, 1983; *see also* Genbank HBV-AF090841). As mentioned before, the promoter and polyadenylation sequences of the E1 expression constructs described in this invention may be derived from other sources without departing from the invention. Also, other functional fragments of the hPGK and HBVpA sequences mentioned above may be used.

[0011] The functionality of pIG270 was shown by transformation of primary Baby Rat Kidney cells (BRK). Comparison with an equivalent Ad5-E1 expression construct taught that Ad35-E1 genes were less efficient in transforming these cells. The same has been found for the E1 genes of Ad12 (Bernards *et al.*, 1982).

[0012] It is unclear which E1 protein(s) determine(s) the difference in transformation efficiency of E1 sequences observed for adenoviruses from different subgroups. In the case of Ad12, transfection studies with chimeric E1A/E1B genes suggested that the efficiency of transformation of BRK cells was determined by the E1A proteins (Bernards *et al.*, 1982). The E1B-55K protein is shown *infra* to contain serotype-specific functions necessary for complementation of E1-deleted adenoviruses. If these functions are related to the regulation of mRNA distribution or another late viral function, it is unlikely that these are involved in the transformation efficiency.

[0013] Analysis of functional domains in the Ad2 or Ad5 E1B-55K proteins using insertion mutants have revealed that functions related to viral replication, late protein synthesis and host protein shut-off are not confined to specific domains but are distributed along the protein (Yew *et al.*, 1990). Using the same set of mutants, the domains important for interaction with p53 and E4-Orf6 were found to be more restricted. In addition to one common binding region (amino acids 262 to 326), p53 binding was affected by mutations at aa 180 and E4-Orf6 binding was affected by mutations at aa 143 (Yew and Berk, 1992; Rubenwolf *et al.*, 1997).

[0014] Altogether these results indicate that it is difficult to separate the E1B-55K functions related to transformation (p53 binding) and late protein synthesis (Orf6 binding).

[0015] The invention discloses new E1 constructs that combine the high efficiency of transformation of one serotype with the serotype-specific complementation function of another serotype. These new constructs are used to transform primary human embryonic retinoblast cells and human amniocytes.

[0016] In another aspect of the invention, the transforming E1 sequences are derived from different serotypes. As disclosed in European Patent application 00201738.2, Ad35E1 sequences are capable of transforming Baby Rat Kidney (BRK) cells, albeit with a lower efficiency than that seen with Ad5-E1 sequences. This was also observed for E1 sequences from Ad12 (Bernards *et al.*, 1982). Therefore, in this aspect of the invention, primary diploid human cells or derivatives thereof are transformed with chimeric E1 construct that consists of part of the E1 sequences of a serotype that enables efficient transformation of primary human cells or derivatives thereof and part of the E1 sequences of another serotype which E1 sequences provide the serotype-specific E1B function(s) that enable(s) efficient propagation of E1-deleted viruses of that serotype. In a preferred embodiment of this aspect of the invention, the E1A region is derived from a subgroup C adenovirus like, but not limited to, Ad5, and the E1B coding sequences are derived from an alternative adenovirus, more particularly from an adenovirus of subgroup B, even more particularly from adenovirus type 35. E1B-21K coding sequences may also be chimeric comprising both subgroup C and subgroup B coding sequences. Preferably, all or most of E1B-21K comprises subgroup C coding sequences. In a more preferred embodiment, the E1A coding sequences and the E1B-21K coding sequences are derived from a subgroup C adenovirus, like, but not limited to, Ad5. In one embodiment the cell further comprises E1B-55k coding sequences that are, preferably, as far as not overlapping with the 21K coding sequences-derived from an adenovirus of subgroup B, more particularly from adenovirus type 35. In an even more preferred embodiment, all E1 coding sequences are derived from a subgroup C adenovirus, like but not limited to Ad5, except for at least the part of the E1B-55K coding sequences that are necessary for serotype-specific complementation of an alternative adenovirus subgroup, more

particularly adenovirus subgroup B, even more particular adenovirus type 35. The invention also provides a packaging cell line wherein the primary, diploid human cells or derivatives thereof have been transformed with a chimeric adenovirus E1 construct comprising part of a first adenovirus E1 coding sequence of a first adenovirus serotype that enables efficient transformation of primary human cells and derivatives thereof; and part of a second adenovirus E1 coding sequence of a second adenovirus serotype, wherein the second adenovirus E1 coding sequence provides the serotype-specific adenovirus E1B function(s) that enable(s) efficient propagation of recombinant adenovirus E1-deleted viruses of the second adenovirus serotype. Preferably, the first adenovirus serotype is a subgroup C adenovirus and the second adenovirus serotype is a subgroup B adenovirus, more particular adenovirus type 35. In one embodiment the packing cell line of the invention comprises bovine adenovirus E1B-55k. Such a bovine E1B-55k expressing cell line is particularly suited for obtaining high yields of a complemented bovine recombinant adenovirus.

[0017] The primary diploid human cells or derivatives thereof are transformed by adenovirus E1 sequences, either operatively linked on one DNA molecule or located on two separate DNA molecules. In the latter case, one DNA molecule carries at least part of the E1 sequences of the serotype-enabling efficient transformation and the second DNA molecule carries at least part of the sequences necessary for serotype-specific complementation. Also provided is a hybrid construct including E1-sequences of the serotype enabling efficient transformation and E1 sequences of another serotype necessary for serotype-specific complementation. The sequences providing serotype specific complementation may of course also contain further activities contributing to transformation. Preferably, the sequences enabling efficient transformation comprise E1A. Preferably, the sequences and the sequences necessary for serotype specific complementation preferably comprise E1B sequences. More preferably, the sequences enabling efficient transforming comprise E1A and E1B-21K sequences and the sequences necessary for serotype specific complementation comprise E1B-55K sequences. Also provided are cells transformed by such hybrid construct. Such cells can favorably be used for the propagation of recombinant E1 deleted adenovirus of another serotype. Of course, it is also possible to provide both functions of E1 sequences on separate constructs. In all



aspects, the sequences are operatively linked to regulatory sequences enabling transcription and translation of the encoded proteins. Preferably, a packaging cell of the invention further comprises a DNA encoding at least E4-orf6 of an adenovirus of subgroup B, preferably adenovirus serotype 35. Preferably, the E4-orf6 is derived from the another serotype. Preferably, the cell comprises E1B-55K and E4-orf6 of the same serotype as the recombinant vector to be propagated/complemented or otherwise produced.

[0018] In another aspect of the invention, new packaging cells are described that are derived from PER.C6 (ECACC deposit number 96022940; Fallaux *et al.*, 1998) and contain Ad35-E1 sequences integrated into their genome. These Ad35-E1 sequences are present in a functional expression cassette, but preferably do not contain sequences overlapping with sequences present in the recombinant viral vector. Preferably, the functional expression cassette consists of a heterologous promoter and poly-adenylation signal functionally linked to Ad35-E1 sequences. More specifically, the Ad35-E1 coding sequences are functionally linked to the human phosphoglycerate gene promoter (hPGK) and hepatitis B virus poly-adenylation signal (HBV-pA). Preferably, Ad35-E1 coding sequences comprise the coding regions of the E1A proteins and the E1B promoter sequences linked to E1B coding sequences up to and including the stop codon of the E1B 55K protein. More preferably, the Ad35-E1 sequences comprise nucleotide 468 to nucleotide 3400 of the Ad35 wt sequence. To be able to select for transfected cells, a dominant selection marker like, but not limited to, the neo<sup>r</sup> gene has to be incorporated on the expression vector or the Ad35 expression vector is cotransfected with a separate expression vector mediating expression of the selection marker. In both cases, the selection marker becomes integrated in the cellular genome. Other Ad5-E1 transformed cell lines like 293 (Graham *et al.*, 1977) and 911 (Fallaux *et al.*, 1996) or established human cell lines like A549 cells may be used without departing from the present invention.

[0019] In another aspect of the invention, PER.C6-derived cells are described that express functional Ad35-E1B sequences. In one embodiment, the Ad35-E1B coding sequences are driven by the E1B promoter and terminated by a heterologous poly-adenylation signal like, but not limited to, the HBVpA. In a preferred embodiment, the

Ad35-E1B coding sequences are driven by a heterologous promoter like, but not limited to, the hPGK promoter or Elongation Factor-1 $\alpha$  (EF-1 $\alpha$ ) promoter and terminated by a heterologous pA signal like, but not limited to, the HBVpA. These Ad35-E1B sequences preferably comprise the coding regions of the E1B-21K and the E1B-55K proteins located between nucleotides 1611 and 3400 of the wild-type (wt) Ad35 sequence. More preferably, the Ad35-E1B sequences comprise nucleotides 1550 to 3400 of the wt Ad35 sequence. In an even more preferred embodiment, the E1B sequences comprise the coding sequences of the E1B-55K gene located between nucleotides 1916 and 3400 of the wt Ad35 sequence. In an even more preferred embodiment a packaging cell line or a cell line of the invention lacks a functional coding sequence for E1B 21k. Such cell lines, in general, produce significantly more recombinant adenovirus than E1B 21K positive cell lines.

[0020] The invention further provides a method for complementing a recombinant adenovirus comprising providing a packaging cell line or a cell line according to the invention, with the recombinant adenovirus and culturing the cell to allow for complementation. In a preferred embodiment the method further comprises harvesting complemented recombinant adenovirus. Preferably, the recombinant adenovirus is derived from adenovirus subgroup B. More preferably, the recombinant adenovirus is derived from adenovirus serotype 35.

[0021] In another aspect, the invention provides a recombinant adenovirus obtained by a method of the invention or with a packaging cell of the invention. Such an adenovirus can be obtained essentially free from contaminating wild type adenovirus, or replication competent adenovirus. Such recombinant adenovirus preparations are very suited for administration of therapeutic sequences to somatic tissues *in vivo* in for instance a gene therapeutic setting. Preferred are recombinant adenoviruses comprising a deletion of nucleic acid encoding at least one E1-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least one E3-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least one E4-region protein. Preferably, such adenovirus further comprises a deletion of nucleic acid encoding at least E4-Orf6 protein. For this reason, the invention also

provides the use of a recombinant adenovirus of the invention for the preparation of a medicament.

[0022] With the term E1B-55K protein as used herein, is meant the protein encoded by the E1B-region in an adenovirus serotype having a similar function in the serotype as provided by the E1B-55K protein Ad5.

[0023] With the term E1B-21K protein as used herein, is meant the protein enclosed by the E1B-region in an adenovirus serotype having a similar function in the serotype as provided by the E1B-19K protein of Ad5. The same terminology applies for the sequences encoding these proteins. When referring to Ad35-E1 sequences from a specified nucleotide to nucleotide 3400 is meant 'up to and including nucleotide 3400'.

[0024] Cell lines subject of this invention are useful for, among other things, the production of recombinant adenoviruses designed for gene therapy and vaccination. The cell lines, being derived from cells of human origin, are also useful for the production of human recombinant therapeutic proteins like, but not limited to human growth factors, human antibodies. In addition the cell lines are useful for the production of human viruses other than adenovirus like, but not limited to, influenza virus, herpes simplex virus, rotavirus, measles virus.

[0025] A preferred derivative of primary, diploid human cells is the PER.C6 cell line (ECACC deposit number 960022940).

[0026] It is within the skills of the artisan to provide for proteins having a similar function in kind as the adenovirus E1 protein referred to in this document. For instance a functional part may be provided and/or a derivative may be provided with a similar function in kind, not necessarily in amount.

[0027] Such parts and derivatives are considered to be part of the invention, in as far as similar transforming/complementing and/or serotype specificity function is provided in kind, not necessarily in amount.

#### BRIEF DESCRIPTION OF THE FIGURES

[0028] FIG. 1: Bar graph showing the percentage of serum samples positive for neutralization for each human wt adenovirus tested (*see*, Example 1 for description of the neutralization assay).

[0029] FIG. 2: Graph showing absence of correlation between the VP/CCID50 ratio and the percentage of neutralization.

[0030] FIG. 3: Bar graph presenting the percentage sera samples that show neutralizing activity to a selection of adenovirus serotypes. Sera were derived from healthy volunteers from Belgium and the UK.

[0031] FIG. 4: Bar graph presenting the percentage sera samples that show neutralizing activity to adenovirus serotypes 5, 11, 26, 34, 35, 48 and 49. Sera were derived from five different locations in Europe and the United States.

[0032] FIG. 5: Map of pAdApt35IP1.

[0033] FIG. 6: Schematic representation of the steps undertaken to construct pWE.Ad35.pIX-rITR.

[0034] FIG. 7: Map of pWE.Ad35.pIX-rITR.

[0035] FIG. 8: Map of pRSV.Ad35-E1.

[0036] FIG. 9 Map of pPGKneopA

[0037] FIG. 10: Map of pRSV-Pneo.

[0038] FIG. 11: Map of pRSVhbv.Neo.

[0039] FIG. 12: Map of pIG.E1A.E1B.

[0040] FIG. 13: Map of pIG135.

[0041] FIG. 14: Map of pIG270.

[0042] FIG. 15: Map of pBr.Ad35.leftITR-pIX.

[0043] FIG. 16: Map of pBr.Ad35.leftITR-pIXdE1A.

[0044] FIG. 17: Map of pBr.Ad35.d21K.

[0045] FIG. 18: Map of pBr.Ad35.d55K1.

[0046] FIG. 19: Map of pBr.Ad35DdSM.

[0047] FIG. 20: Schematic representation of Ad35-E1A/E1B deletion constructs.

[0048] FIG. 21: Map of pIG.35BL.

[0049] FIG. 22: Map of pRSVneo4.

[0050] FIG. 23: Map of pIG35Bneo.

[0051] FIG. 24: Map of pIG35.55K.

[0052] FIG. 25: Map of pIG535.

- [0053] FIG. 26: Map of pIG635.  
[0054] FIG. 27: Map of pIG735.  
[0055] FIG. 28: Map of pCC271.  
[0056] FIG. 29: Map of pCC535s.  
[0057] FIG. 30: Map of pCR535E1B.  
[0058] FIG. 31: Map of pCC2155s.  
[0059] FIG. 32: Map of pCC536s.  
[0060] FIG. 33: Map of pIG536.  
[0061] FIG. 34: Map of pBr.Ad35.PRn.  
[0062] FIG. 35: Map of pBr.Ad35.PRnΔE3.  
[0063] FIG. 36: Map of pWE.Ad35.pIX-rITRΔE3.

[0064] FIG. 37: Alignment of E1B-21K amino acid sequences in pCC536s (SEQ ID NO:45), wtAd5 (SEQ ID NO:46) and wtAd35 (SEQ ID NO:47) (A) and E1B-55K amino acid sequences in pCC536s (SEQ ID NO:48), wtAd5 (SEQ ID NO:49) and wtAd35 (SEQ ID NO:50) (B).

#### DETAILED DESCRIPTION OF THE INVENTION

[0065] The invention is further explained by the use of the following illustrative examples.

#### EXAMPLES

##### Example 1

*A high throughput assay for the detection of neutralizing activity in human serum*

[0066] To enable screening of a large amount of human sera for the presence of neutralizing antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.

### *Human sera*

[0067] A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between ages 20 and 60 years old with no restriction for race. All volunteers signed an informed consent form. People professionally involved in adenovirus research were excluded.

[0068] Approximately 60 ml blood was drawn in dry tubes. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20°C until further use.

[0069] Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquoted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity large enough to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. The plates were loaded with eight different sera in duplo (100 µl/well) according to the scheme below:

S1/2	S1/4	S1/8	S1/1 6	S1/3 2	S5/2	S5/4	S5/8	S5/1 6	S5/3 2	-	-
S1/2	S1/4	S1/8	S1/1 6	S1/3 2	S5/2	S5/4	S5/8	S5/1 6	S5/3 2	-	-
S2/2	S2/4	S2/8	S2/1 6	S2/3 2	S6/2	S6/4	S6/8	S6/1 6	S6/3 2	-	-
S2/2	S2/4	S2/8	S2/1 6	S2/3 2	S6/2	S6/4	S6/8	S6/1 6	S6/3 2	-	-
S3/2	S3/4	S3/8	S3/1 6	S3/3 2	S7/2	S7/4	S7/8	S7/1 6	S7/3 2	-	-
S3/2	S3/4	S3/8	S3/1 6	S3/3 2	S7/2	S7/4	S7/8	S7/1 6	S7/3 2	-	-
S4/2	S4/4	S3/8	S3/1 6	S3/3 2	S8/2	S8/4	S8/8	S8/1 6	S8/3 2	-	-
S4/2	S4/4	S3/8	S3/1 6	S3/3 2	S8/2	S8/4	S8/8	S8/1 6	S8/3 2	-	-

Where S1/2 to S8/2 in columns 1 and 6 represent 1 X diluted sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial dilutions. The last plates also contained four wells filled with 100 µl fetal calf serum as a negative control. Plates were kept at -20°C until further use.

### *Preparation of human adenovirus stocks*

[0070] Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (Fallaux *et al.*, 1998) and harvested upon full CPE. After freeze/thawing, 1-2 ml of the crude lysates were used to inoculate a T80 flask with PER.C6 and virus was harvested at full CPE. The timeframe between inoculation and occurrence of CPE, as well as the amount of virus needed to re-infect a new culture, differed between serotypes. Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm<sup>2</sup> three-layer flasks with PER.C6 cells. Upon occurrence of CPE, cells were harvested by tapping the flask, pelleted and virus was isolated and purified by a two-step CsCl gradient as follows. Cell pellets were dissolved in 50 ml 10 mM NaPO<sub>4</sub> buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C, 5.6 ml sodium deoxycholate (5% w/v) was added. The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875 µl 1M MgCl<sub>2</sub> was added. After the addition of 375 µl DNase (10 mg/ml), the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with FREON (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered cesium chloride block gradient (range: 1.2/1.4 g/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continuous gradient of 1.33 g/ml of cesium chloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess cesium chloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 liter PBS supplemented with CaCl<sub>2</sub> (0.9 mM), MgCl<sub>2</sub> (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

[0071] To determine the number of virus particles per milliliter, 50 µl of the virus batch is run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). Viruses were eluted using a NaCl gradient ranging from 0 to 600

mM. As depicted in table I, the NaCl concentration by which the viruses were eluted differed significantly among serotypes.

[0072] Most human adenoviruses replicated well on PER.C6 cells with a few exceptions. Adenovirus type 8 and 40 were grown on 911-E4 cells (He *et al.*, 1998). Purified stocks contained between  $5 \times 10^{10}$  and  $5 \times 10^{12}$  virus particles/ml (VP/ml; see table I).

#### *Titration of purified human adenovirus stocks*

[0073] Adenoviruses were titrated on PER.C6 cells to determine the amount of virus necessary to obtain full CPE in five days, the length of the neutralization assay. Hereto, 100  $\mu$ l medium was dispensed into each well of 96-well plates. 25  $\mu$ l of adenovirus stocks pre-diluted  $10^4$ ,  $10^5$ ,  $10^6$  or  $10^7$  times were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. Then 25  $\mu$ l was brought from column 2 to column 3 and again mixed. This was repeated until column 11, after which 25  $\mu$ l from column 11 was discarded. This way, serial dilutions in steps of 5 were obtained starting off from a pre-diluted stock. Then  $3 \times 10^4$  PER.C6 cells (ECACC deposit number 96022940) were added in a 100  $\mu$ l volume and the plates were incubated at 37 °C, 5% CO<sub>2</sub> for five or six days. CPE was monitored microscopically. The method of Reed and Muensch was used to calculate the cell culture-inhibiting dose 50% (CCID50).

[0074] In parallel, identical plates were set up that were analyzed using the MTT assay (Promega). In this assay, living cells are quantified by colorimetric staining. Hereto, 20  $\mu$ l MTT (7.5 mgr/ml in PBS) was added to the wells and incubated at 37 °C, 5% CO<sub>2</sub> for two hours. The supernatant was removed and 100  $\mu$ l of a 20:1 isopropanol/triton-X100 solution was added to the wells. The plates were put on a 96-well shaker for 3-5 minutes to solubilize the precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). By this assay, wells with proceeding CPE or full CPE can be distinguished.

#### *Neutralization assay*

[0075] 96-well plates with diluted human serum samples were thawed at 37 °C, 5% CO<sub>2</sub>. Adenovirus stocks diluted to 200 CCID50 per 50  $\mu$ l were prepared and 50



μl aliquots were added to columns 1-11 of the plates with serum. Plates were incubated for 1 hour at 37°C, 5% CO<sub>2</sub>. Then, 50 μl PER.C6 cells at 6x10<sup>5</sup>/ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO<sub>2</sub>. Supernatant was removed using fresh pipette tips for each row and 200μl fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 °C, 5% CO<sub>2</sub>. In addition, parallel control plates were set up *in duplo*, with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows A and B and with negative control serum (FCS) in rows C and D. Also, in each of the rows E-H, a titration was performed as described above with steps of five times dilutions starting with 200 CCID<sub>50</sub> of each virus to be tested. On day 5, one of the control plates was analyzed microscopically and with the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, *i.e.*, the first dilution in the control titration experiment analyzed by MTT shows clear cell death, all assay plates were processed. If not, the assay was allowed to proceed for one or more days until full CPE was apparent, after which all plates were processed. In most cases, the assay was terminated at day 5. For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days.

[0076] A serum sample is regarded as “non-neutralizing” when, at the highest serum concentration, a maximum protection of 40% is seen compared to controls without serum.

[0077] The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers are shown in FIG. 1. As expected, the percentage of serum samples that contained neutralizing antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralizing antibodies to Ad35. Also, the number of individuals with neutralizing antibody titers to the serotypes 26, 34 and 48 was very low. Therefore, recombinant E1-deleted adenoviruses based on Ad35 or one of the other above-mentioned serotypes have an important advantage compared to recombinant vectors based on Ad5 with respect to clearance of the viruses by neutralizing antibodies.

[0078] Also, Ad5-based vectors that have parts of the capsid proteins involved in immunogenic response of the host replaced by the corresponding parts of the capsid

proteins of Ad35 or one of the other serotypes will be less, or even not, neutralized by the vast majority of human sera.

[0079] As can be seen in Table I, the VP/CCID50 ratio calculated from the virus particles per ml and the CCID50 obtained for each virus in the experiments was highly variable and ranged from 0.4 to 5 log. This is probably caused by different infection efficiencies of PER.C6 cells and by differences in replication efficiency of the viruses. Furthermore, differences in batch qualities may play a role. A high VP/CCID50 ratio means that more viruses were put in the wells to obtain CPE in 5 days. As a consequence, the outcome of the neutralization study might be biased since more inactive virus particles could shield the antibodies. To check whether this phenomenon had taken place, the VP/CCID50 ratio was plotted against the percentage of serum samples found positive in the assay (FIG. 2). The graph clearly shows that there is no negative correlation between the amount of viruses in the assay and neutralization in serum.

#### Example 2

*The prevalence of neutralizing activity (NA) to Ad35 is low in human sera from different geographic locations*

[0080] In Example 1, the analysis of neutralizing activity ("NA") in human sera from one location in Belgium was described. Strikingly, of a panel of 44 adenovirus serotypes tested, one serotype, Ad35, was not neutralized in any of the 100 sera assayed. In addition, a few serotypes, Ad26, Ad34 and Ad48 were found to be neutralized in 8%, or less, of the sera tested. This analysis was further extended to other serotypes of adenovirus not previously tested and, using a selection of serotypes from the first screen, was also extended to sera from different geographic locations.

[0081] Hereto, adenoviruses were propagated, purified and tested for neutralization in the CPE-inhibition assay as described in Example 1. Using the sera from the same batch as in Example 1, adenovirus serotypes 7B, 11, 14, 18 and 44/1876 were tested for neutralization. These viruses were found to be neutralized in, respectively, 59, 13, 30, 98 and 54 % of the sera. Thus, of this series, Ad11 is neutralized with a relatively low frequency.

[0082] Since it is known that the frequency of isolation of adenovirus serotypes from human tissue, as well as the prevalence of NA to adenovirus serotypes, may differ on different geographic locations, we further tested a selection of the adenovirus serotypes against sera from different places. Human sera were obtained from two additional places in Europe (Bristol, UK and Leiden, NL) and from two places in the United States (Stanford, CA and Great Neck, NY). Adenoviruses that were found to be neutralized in 20% or less of the sera in the first screen, as well as Ad2, Ad5, Ad27, Ad30, Ad38, Ad43, were tested for neutralization in sera from the UK. The results of these experiments are presented in FIG. 3. Adenovirus serotypes 2 and 5 were again neutralized in a high percentage of human sera. Furthermore, some of the serotypes that were neutralized in a low percentage of sera in the first screen are neutralized in a higher percentage of sera from the UK, for example, Ad26 (7% vs. 30%), Ad28 (13% vs. 50%), Ad34 (5% vs. 27%) and Ad48 (8% vs. 32%). Neutralizing activity against Ad11 and Ad49 that were found in a relatively low percentage of sera in the first screen, are found in an even lower percentage of sera in this second screen (13% vs. 5% and 20% vs. 11%, respectively). Serotype Ad35 that was not neutralized in any of the sera in the first screen, was now found to be neutralized in a low percentage (8%) of sera from the UK. The prevalence of NA in human sera from the UK is the lowest to serotypes Ad11 and Ad35.

[0083] For further analysis, sera was obtained from two locations in the US (Stanford, CA and Great Neck, NY) and from The Netherlands (Leiden). FIG. 4 presents an overview of data obtained with these sera and the previous data. Not all viruses were tested in all sera, except for Ad5, Ad11 and Ad35. The overall conclusion from this comprehensive screen of human sera is that the prevalence of neutralizing activity to Ad35 is the lowest of all serotypes throughout the western countries: on average 7% of the human sera contain neutralizing activity (5 different locations). Another B-group adenovirus, Ad11 is also neutralized in a low percentage of human sera (average 11% in sera from 5 different locations). Adenovirus type 5 is neutralized in 56% of the human sera obtained from 5 different locations. Although not tested in all sera, D-group serotype 49 is also neutralized with relatively low frequency in samples from Europe and from one location of the US (average 14%).

[0084] In the herein described neutralization experiments, a serum is judged non-neutralizing when, in the well with the highest serum concentration, the maximum protection of CPE is 40% compared to the controls without serum. The protection is calculated as follows:

$$1\% \text{ protection} = \frac{\text{OD corresponding well} - \text{OD virus control}}{\text{OD non-infected control} - \text{OD virus control}} \times 100 \%$$

[0085] As described in Example 1, the serum is plated in five different dilutions ranging from 4x to 64x diluted. Therefore, it is possible to distinguish between low titers (*i.e.*, neutralization only in the highest serum concentrations) and high titers of NA (*i.e.*, also neutralization in wells with the lowest serum concentration). Of the human sera used in our screen that were found to contain neutralizing activity to Ad5, 70% turned out to have high titers, whereas, of the sera that contained NA to Ad35, only 15% had high titers. Of the sera that were positive for NA to Ad11, only 8% had high titers. For Ad49, this was 5%. Therefore, not only is the frequency of NA to Ad35, Ad11 and Ad49 much lower as compared to Ad5, but of the sera that do contain NA to these viruses, the vast majority have low titers. Adenoviral vectors based on Ad11, Ad35 or Ad49 have, therefore, a clear advantage over Ad5-based vectors when used as gene therapy vehicles or vaccination vectors *in vivo* or in any application where infection efficiency is hampered by neutralizing activity.

[0086] In the following examples, the construction of a vector system for the generation of safe, RCA-free Ad35-based vectors is described.

### Example 3

#### *Sequence of the human adenovirus type 35*

[0087] Ad35 viruses were propagated on PER.C6 cells and DNA was isolated as follows: To 100 µl of virus stock (Ad35:  $3.26 \times 10^{12}$  VP/ml), 10µl 10X DNase buffer (130 mM Tris-HCl pH7.5; 1,2 M  $\text{CaCl}_2$ ; 50mM  $\text{MgCl}_2$ ) was added. After addition of 10 µl 10mgr/ml DNase I (Roche Diagnostics), the mixture was incubated for 1 hr. at 37°C. Following addition of 2.5µl 0.5M EDTA, 3.2µl 20% SDS and 1.5µl ProteinaseK (Roche

Diagnostics; 20mgr/ml), samples were incubated at 50°C for 1 hr. Next, the viral DNA was isolated using the GENE CLEAN spin kit (Bio101 Inc.) according to the manufacturer's instructions. DNA was eluted from the spin column with 25 µl sterile MilliQ water. The total sequence was generated by Qiagen Sequence Services (Qiagen GmbH, Germany). Total viral DNA was sheared by sonification and the ends of the DNA were made blunt by T4 DNA polymerase. Sheared blunt fragments were size fractionated on agarose gels and gel slices corresponding to DNA fragments of 1.8 to 2.2kb were obtained. DNA was purified from the gel slices by the QIAquick gel extraction protocol and subcloned into a shotgun library of pUC19 plasmid cloning vectors. An array of clones in 96-well plates covering the target DNA 8 (+/- 2) times was used to generate the total sequence. Sequencing was performed on Perkin-Elmer 9700 thermocyclers using Big Dye Terminator chemistry and AmpliTaq FS DNA polymerase followed by purification of sequencing reactions using QIAGEN DyeEx 96 technology. Sequencing reaction products were then subjected to automated separation and detection of fragments on ABI 377 XL 96 lane sequencers. Initial sequence results were used to generate a contiguous sequence and gaps were filled in by primer walking reads on the target DNA or by direct sequencing of PCR products. The ends of the virus turned out to be absent in the shotgun library, most probably due to cloning difficulties resulting from the amino acids of pTP that remain bound to the ITR sequences after proteinase K digestion of the viral DNA. Additional sequence runs on viral DNA solved most of the sequence in those regions, however, it was difficult to obtain a clear sequence of the most terminal nucleotides. At the 5' end the sequence portion obtained was 5'-CCAATAATATACCT-3' (SEQ ID NO:1) while at the 3' end, the obtained sequence portion was 5'-AGGTATATTATTGATGATGGG-3' (SEQ ID NO:2). Most human adenoviruses have a terminal sequence 5'-CATCATCAATAATATACC-3' (SEQ ID NO:3). In addition, a clone representing the 3' end of the Ad35 DNA obtained after cloning the terminal 7 kb Ad35 EcoRI fragment into pBr322 also turned out to have the typical CATCATCAATAAT... sequence. Therefore, Ad35 may have the typical end sequence and the differences obtained in sequencing directly on the viral DNA are due to artifacts correlated with run-off sequence runs and the presence of residual amino acids of pTP.

[0088] The total sequence of Ad35 with corrected terminal sequences is given in SEQ ID NO:44. Based sequence homology with Ad5 (Genbank # M72360) and Ad7 (partial sequence Genbank # X03000) and on the location of open reading frames, the organization of the virus is identical to the general organization of most human adenoviruses, especially the subgroup B viruses. The total length of the genome is 34,794 basepairs.

#### Example 4

*Construction of a plasmid-based vector system to generate recombinant Ad35-based viruses.*

[0089] A functional plasmid-based vector system to generate recombinant adenoviral vectors comprises the following components:

1. An adapter plasmid comprising a left ITR and packaging sequences derived from Ad35 and at least one restriction site for insertion of a heterologous expression cassette and lacking E1 sequences. Furthermore, the adapter plasmid contains Ad35 sequences 3' from the E1B coding region including the pIX promoter and coding sequences enough to mediate homologous recombination of the adapter plasmid with a second nucleic acid molecule.
2. A second nucleic acid molecule, comprising sequences homologous to the adapter plasmid, and Ad35 sequences necessary for the replication and packaging of the recombinant virus, that is, early, intermediate and late genes that are not present in the packaging cell.
3. A packaging cell providing at least functional E1 proteins capable of complementing the E1 function of Ad35.

[0090] Other methods for generating recombinant adenoviruses on complementing packaging cells are known in the art and may be applied to Ad35 viruses without departing from the invention. As an example, the construction of a plasmid-based system, as outlined above, is described in detail below.

1) Construction of Ad35 adapter plasmids.

[0012] The adapter plasmid pAdApt (described in International Patent Application WO99/55132) was first modified to obtain adapter plasmids that contain extended polylinkers and that have convenient unique restriction sites flanking the left ITR and the adenovirus sequence at the 3' end to enable liberation of the adenovirus insert from plasmid vector sequences. Construction of these plasmids is described below in detail:

[0092] Adapter plasmid pAdApt was digested with SalI and treated with Shrimp Alkaline Phosphatase to reduce religation. A linker, composed of the following two phosphorylated and annealed oligos: ExSalPacF 5' – TCG ATG GCA AAC AGC TAT TAT GGG TAT TAT GGG TTC GAA TTA ATT AA- 3' (SEQ ID NO:4) and ExSalPacR 5' – TCG ATT AAT TAA TTC GAA CCC ATA ATA CCC ATA ATA GCT GTT TGC CA- 3' (SEQ ID NO:5) was directly ligated into the digested construct, thereby replacing the SalI restriction site by Pi-PspI, SwaI and PacI. This construct was designated pADAPT+ExSalPac linker. Furthermore, part of the left ITR of pAdApt was amplified by PCR using the following primers: PCLIPMSF: 5'- CCC CAA TTG GTC GAC CAT CAT CAA TAA TAT ACC TTA TTT TGG -3' (SEQ ID NO:6) and pCLIPBSRG1: 5'- GCG AAA ATT GTC ACT TCC TGT G - 3' (SEQ ID NO:7)). The amplified fragment was digested with MunI and BsrGI and cloned into pAd5/Clip (described in International Patent Application WO99/55132), which was partially digested with EcoRI and after purification digested with BsrGI, thereby re-inserting the left ITR and packaging signal. After restriction enzyme analysis, the construct was digested with ScaI and SgrAI and an 800 bp fragment was isolated from gel and ligated into ScaI/SgrAI digested pADAPT+ExSalPac linker. The resulting construct, designated pIPspSalAdapt, was digested with SalI, dephosphorylated, and ligated to the phosphorylated ExSalPacF/ExSalPacR double-stranded linker previously mentioned. A clone in which the PacI site was closest to the ITR was identified by restriction analysis and sequences were confirmed by sequence analysis. This novel pAdApt construct, termed pIPspAdapt, thus harbours two ExSalPac linkers containing recognition sequences for PacI, PI-PspI and BstBI, which surround the adenoviral part of the

adenoviral adapter construct, and which can be used to linearize the plasmid DNA prior to co-transfection with adenoviral helper fragments.

**[0093]** In order to further increase transgene cloning permutations, a number of polylinker variants were constructed based on pIPspAdapt. For this purpose, pIPspAdapt was first digested with EcoRI and dephosphorylated. A linker composed of the following two phosphorylated and annealed oligos: Ecolinker+: 5' –AAT TCG GCG CGC CGT CGA CGA TAT CGA TAG CGG CCG C –3' (SEQ ID NO:8) and Ecolinker: 5' –AAT TGC GGC CGC TAT CGA TAT CGT CGA CGG CGC GCC G –3' (SEQ ID NO:9) was ligated into this construct, thereby creating restriction sites for AscI, SalI, EcoRV, ClaI and NotI. Both orientations of this linker were obtained, and sequences were confirmed by restriction analysis and sequence analysis. The plasmid containing the polylinker in the order 5' HindIII, KpnI, AgeI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt1, while the plasmid containing the polylinker in the order HindIII, KpnI, AgeI, NotI, ClaI, EcoRV, SalI, AscI, EcoRI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt2.

**[0094]** To facilitate the cloning of other sense or antisense constructs, a linker composed of the following two oligonucleotides was designed to reverse the polylinker of pIPspAdapt: HindXba+ 5'-AGC TCT AGA GGA TCC GTT AAC GCT AGC GAA TTC ACC GGT ACC AAG CTT A-3' (SEQ ID NO:10); HindXba- 5'-CTA GTA AGC TTG GTA CCG GTG AAT TCG CTA GCG TTA ACG GAT CCT CTA G-3' (SEQ ID NO:11). This linker was ligated into HindIII/XbaI digested pIPspAdapt and the correct construct was isolated. Confirmation was done by restriction enzyme analysis and sequencing. This new construct, pIPspAdaptA, was digested with EcoRI and the previously mentioned Ecolinker was ligated into this construct. Both orientations of this linker were obtained, resulting in pIPspAdapt3, which contains the polylinker in the order XbaI, BamHI, HpaI, NheI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, AgeI, KpnI and HindIII. All sequences were confirmed by restriction enzyme analysis and sequencing.

**[0095]** Adapter plasmids based on Ad35 were then constructed as follows:

**[0096]** The left ITR and packaging sequence corresponding to Ad35 wt sequences nucleotides 1 to 464 (SEQ ID NO:44) were amplified by PCR on wt Ad35 DNA using the following primers:



Primer 35F1:

5'-CGG AAT TCT TAA TTA ATC GAC ATC ATC AAT AAT ATA CCT TAT AG-3'  
(SEQ ID NO:12)

Primer 35R2:

5'-GGT GGT CCT AGG CTG ACA CCT ACG TAA AAA CAG-3' (SEQ ID NO:13)

Amplification introduces a PacI site at the 5' end and an AvrII site at the 3' end of the sequence.

[0097] For the amplification, Platinum Pfx DNA polymerase enzyme (LTI) was used according to manufacturer's instructions, but with primers at 0.6  $\mu$ M and with DMSO added to a final concentration of 3%. Amplification program was as follows: 2 min. at 94°C, (30 sec. 94°C, 30 sec. at 56°C, 1 min. at 68°C) for 30 cycles, followed by 10 min. at 68°C.

[0098] The PCR product was purified using a PCR purification kit (LTI) according to the manufacturer's instructions and digested with PacI and AvrII. The digested fragment was then purified from gel using the GENECLAN kit (Bio 101, Inc.). The Ad5-based adapter plasmid pIPspAdApt-3 was digested with AvrII and then partially with PacI and the 5762 bp fragment was isolated in an LMP agarose gel slice and ligated with the above-mentioned PCR fragment digested with the same enzymes and transformed into electrocompetent DH10B cells (LTI). The resulting clone is designated pIPspAdApt3-Ad35LTR.

[0099] In parallel, a second piece of Ad35 DNA was amplified using the following primers:

35F3: 5'- TGG TGG AGA TCT GGT GAG TAT TGG GAA AAC-3' (SEQ ID NO:14)

35R4: 5'- CGG AAT TCT TAA TTA AGG GAA ATG CAA ATC TGT GAG G-3'  
(SEQ ID NO:15)

[0100] The sequence of this fragment corresponds to nucleotides 3401 to 4669 of wt Ad35 (SEQ ID NO:44) and contains 1.3kb of sequences starting directly 3' from the E1B-55k coding sequence. Amplification and purification were done as previously described herein for the fragment containing the left ITR and packaging sequence. The PCR fragment was then digested with PacI and subcloned into pNEB193 vector (New England Biolabs) digested with SmaI and PacI. The integrity of the

sequence of the resulting clone was checked by sequence analysis. pNEB/Ad35pF3R4 was then digested with BglII and PacI and the Ad35 insert was isolated from gel using the QIAExII kit (Qiagen). pIPspAdApt3-Ad35lITR was digested with BglII and then partially with PacI. The 3624 bp fragment (containing vector sequences, the Ad35 ITR and packaging sequences as well as the CMV promoter, multiple cloning region and polyA signal) was also isolated using the QIAExII kit (Qiagen). Both fragments were ligated and transformed into competent DH10B cells (LTI). The resulting clone, pAdApt35IP3, has the expression cassette from pIPspAdApt3 but contains the Ad35 left ITR and packaging sequences and a second fragment corresponding to nucleotides 3401 to 4669 from Ad35. A second version of the Ad35 adapter plasmid having the multiple cloning site in the opposite orientation was made as follows:

[0101] pIPspAdapt1 was digested with NdeI and BglII and the 0.7 kbp band containing part of the CMV promoter, the MCS and SV40 polyA was isolated and inserted in the corresponding sites of pAdApt35IP3 generating pAdApt35IP1 (Fig. 5).

[0102] pAdApt35.LacZ and pAdApt35.Luc adapter plasmids were then generated by inserting the transgenes from pcDNA.LacZ (digested with KpnI and BamHI) and pAdApt.Luc (digested with HindIII and BamHI) into the corresponding sites in pAdApt35IP1. The generation of pcDNA.LacZ and pAdApt.Luc is described in International Patent Application WO99/55132.

## 2) Construction of cosmid pWE.Ad35.pIX-rITR

[0103] FIG. 6 presents the various steps undertaken to construct the cosmid clone containing Ad35 sequences from bp 3401 to 34794 (end of the right ITR) that are described in detail below.

[0104] A first PCR fragment (pIX-NdeI) was generated using the following primer set:

35F5: 5'-CGG AAT TCG CGG CCG CGG TGA GTA TTG GGA AAA C -3' (SEQ ID NO:16)

35R6: 5'-CGC CAG ATC GTC TAC AGA ACA G-3' (SEQ ID NO:17)

[0105] DNA polymerase Pwo (Roche) was used according to manufacturer's instructions, however, with an end concentration of 0.6  $\mu$ M of both primers and using 50

ngr wt Ad35 DNA as template. Amplification was done as follows: 2 min. at 94 °C, 30 cycles of 30 sec. at 94 °C, 30 sec. at 65 °C and 1 min. 45 sec. at 72 °C, followed by 8 min. at 68 °C. To enable cloning in the TA cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase (HT Biotechnology LTD) for 10 min. at 72 °C was performed.

**[0106]** The 3370 bp amplified fragment contains Ad35 sequences from bp 3401 to 6772 with a NotI site added to the 5' end. Fragments were purified using the PCR purification kit (LTI).

**[0107]** A second PCR fragment (NdeI-rITR) was generated using the following primers:

35F7: 5'-GAA TGC TGG CTT CAG TTG TAA TC -3' (SEQ ID NO:18)

35R8: 5'- CGG AAT TCG CGG CCG CAT TTA AAT CAT CAT CAA TAA TAT ACC-3' (SEQ ID NO:19)

**[0108]** Amplification was done with pfx DNA polymerase (LTI) according to manufacturer's instructions but with 0.6 µM of both primers and 3% DMSO using 10 ngr. of wt Ad35 DNA as template. The program was as follows: 3 min. at 94 °C and 5 cycles of 30 sec. at 94 °C, 45 sec. at 40 °C, 2 min.45 sec. at 68 °C followed by 25 cycles of 30 sec. at 94 °C, 30 sec. at 60 °C, 2 min.45 sec. at 68 °C. To enable cloning in the TA-cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase for 10 min. at 72 °C was performed. The 1.6 kb amplified fragment ranging from nucleotides 33178 to the end of the right ITR of Ad35, was purified using the PCR purification kit ( LTI).

**[0109]** Both purified PCR fragments were ligated into the PCR2.1 vector of the TA-cloning kit (Invitrogen) and transformed into STBL-2 competent cells (LTI). Clones containing the expected insert were sequenced to confirm correct amplification. Next, both fragments were excised from the vector by digestion with NotI and NdeI and purified from gel using the GENECLAN kit (BIO 101, Inc.). Cosmid vector pWE15 (Clontech) was digested with NotI, dephosphorylated and also purified from gel. These three fragments were ligated and transformed into STBL2 competent cells (LTI). One of the correct clones that contained both PCR fragments was then digested with NdeI, and the linear fragment was purified from gel using the GENECLAN kit. Ad35 wt DNA was digested with NdeI and the 26.6 kb fragment was purified from LMP gel using agarase enzyme (Roche) according to the manufacturer's instructions. These fragments

were ligated together and packaged using  $\lambda$ 1 phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into STBL-2 cells, colonies were grown on plates and analysed for presence of the complete insert. One clone with the large fragment inserted in the correct orientation and having the correct restriction patterns after independent digestions with three enzymes (NcoI, PvuII and ScaI) was selected. This clone is designated pWE.Ad35.pIX-rITR. It contains the Ad35 sequences from bp 3401 to the end and is flanked by NotI sites (FIG. 7).

3) Generation of Ad35-based recombinant viruses on PER.C6.

[0110] Wild type Ad35 virus can be grown on PER.C6 packaging cells to very high titers. However, whether the Ad5-E1 region that is present in PER.C6 is able to complement E1-deleted Ad35 recombinant viruses is unknown. To test this, PER.C6 cells were cotransfected with the above-described adapter plasmid pAdApt35.LacZ and the large backbone fragment pWE.Ad35.pIX-rITR. First, pAdApt35.LacZ was digested with PacI and pWE.Ad35.pIX-rITR was digested with NotI. Without further purification, 4 µgr of each construct was mixed with DMEM (LTI) and transfected into PER.C6 cells, seeded at a density of  $5 \times 10^6$  cells in a T25 flask the day before, using Lipofectamin (LTI) according to the manufacturer's instructions. As a positive control, 6µgr of PacI digested pWE.Ad35.pIX-rITR DNA was cotransfected with a 6.7 kb NheI fragment isolated from Ad35 wt DNA containing the left end of the viral genome including the E1 region. The next day, medium (DMEM with 10% FBS and 10mM MgCl<sub>2</sub>) was refreshed and cells were further incubated. At day 2 following the transfection, cells were trypsinized and transferred to T80 flasks. The positive control flask showed CPE at five days following transfection, showing that the pWE.Ad35.pIX-rITR construct is functional, at least in the presence of Ad35-E1 proteins. The transfection with the Ad35 LacZ adapter plasmid and pWE.Ad35.pIX-rITR did not give rise to CPE. These cells were harvested in the medium at day 10 and freeze/thawed once to release virus from the cells. 4 ml of the harvested material was added to a T80 flask with PER.C6 cells (at 80% confluency) and incubated for another five days. This harvest/re-infection was repeated two times but there was no evidence for virus associated CPE.

[0111] From this experiment, it seems that the Ad5-E1 proteins are not, or not well enough, capable of complementing Ad35 recombinant viruses. However, it may be that the sequence overlap of the adapter plasmid and the pWE.Ad35.pIX-rITR backbone plasmid is not large enough to efficiently recombine and give rise to a recombinant virus genome. The positive control transfection was done with a 6.7kb left end fragment and, therefore, the sequence overlap was about 3.5kb. The adapter plasmid and the pWE.Ad35.pIX-rITR fragment have a sequence overlap of 1.3kb. To check whether the sequence overlap of 1.3 kb is too small for efficient homologous recombination, a co-transfection was done with PacI digested pWE.Ad35.pIX-rITR and a PCR fragment of Ad35 wt DNA generated with the above-mentioned 35F1 and 35R4 using the same

procedures as previously described herein. The PCR fragment thus contains left end sequences up to bp 4669 and, therefore, has the same overlap sequences with pWE.Ad35.pIX-rITR as the adapter plasmid pAdApt35.LacZ, but has Ad35-E1 sequences. Following PCR column purification, the DNA was digested with Sall to remove possible intact template sequences. A transfection with the digested PCR product alone served as a negative control. Four days after the transfection, CPE occurred in the cells transfected with the PCR product and the Ad35 pIX-rITR fragment, and not in the negative control. This result shows that a 1.3kb overlapping sequence is sufficient to generate viruses in the presence of Ad35-E1 proteins. From these experiments, we conclude that the presence of at least one of the Ad35-E1 proteins is necessary to generate recombinant Ad35 based vectors from plasmid DNA on Ad5 complementing cell lines.

#### Example 5

##### 1) Construction of Ad35-E1 expression plasmids

[0112] Since Ad5-E1 proteins in PER.C6 are incapable of complementing Ad35 recombinant viruses efficiently, Ad35-E1 proteins have to be expressed in Ad5 complementing cells (*e.g.*, PER.C6). Alternatively, a new packaging cell line expressing Ad35-E1 proteins has to be made, starting from either diploid primary human cells or established cell lines not expressing adenovirus E1 proteins. To address the first possibility, the Ad35-E1 region was cloned in expression plasmids as described below.

[0113] First, the Ad35-E1 region from bp 468 to bp 3400 was amplified from wt Ad35 DNA using the following primer set:

35F11: 5'-GGG GTA CCG AAT TCT CGC TAG GGT ATT TAT ACC-3' (SEQ ID NO:20)

35F10: 5'-GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3' (SEQ ID NO:21)

[0114] This PCR introduces a KpnI and EcoRI site at the 5' end and an SbfI and XbaI site at the 3' end.

[0115] Amplification on 5 ngr. template DNA was done with Pwo DNA polymerase (Roche) using the manufacturer's instructions, however, with both primers at

a final concentration of 0.6  $\mu$ M. The program was as follows: 2 min. at 94 °C, 5 cycles of 30 sec. at 94 °C, 30 sec. at 56 °C and 2 min. at 72 °C, followed by 25 cycles of 30 sec. at 94°C, 30 sec. at 60 °C and 2 min. at 72 °C, followed by 10 min. at 72 °C. PCR product was purified by a PCR purification kit (LTI) and digested with KpnI and XbaI. The digested PCR fragment was then ligated to the expression vector pRSVhbmNeo (see below) also digested with KpnI and XbaI. Ligations were transformed into competent STBL-2 cells (LTI) according to manufacturer's instructions and colonies were analysed for the correct insertion of Ad35-E1 sequences into the polylinker in between the RSV promoter and HBV polyA.

[0116] The resulting clone was designated pRSV.Ad35-E1 (FIG. 8). The Ad35 sequences in pRSV.Ad35-E1 were checked by sequence analysis.

[0117] pRSVhbmNeo was generated as follows: pRc-RSV (Invitrogen) was digested with PvuII, dephosphorylated with TSAP enzyme (LTI), and the 3kb vector fragment was isolated in low melting point agarose (LMP). Plasmid pPGKneoA (FIG. 9; described in International Patent Application WO96/35798) was digested with SspI completely to linearize the plasmid and facilitate partial digestion with PvuII. Following the partial digestion with PvuII, the resulting fragments were separated on a LMP agarose gel and the 2245 bp PvuII fragment, containing the PGK promoter, neomycin-resistance gene and HBVpolyA, was isolated. Both isolated fragments were ligated to give the expression vector pRSV-pNeo that now has the original SV40prom-neo-SV40polyA expression cassette replaced by a PGKprom-neo-HBVpolyA cassette (FIG. 10). This plasmid was further modified to replace the BGHpA with the HBVpA as follows: pRSVpNeo was linearised with ScaI and further digested with XbaI. The 1145 bp fragment, containing part of the Amp gene and the RSV promoter sequences and polylinker sequence, was isolated from gel using the GENECLAN kit (Bio Inc. 101). Next, pRSVpNeo was linearised with ScaI and further digested partially with EcoRI and the 3704 bp fragment containing the PGKneo cassette and the vector sequences were isolated from gel as above. A third fragment, containing the HBV polyA sequence flanked by XbaI and EcoRI at the 5' and 3' end, respectively, was then generated by PCR amplification on pRSVpNeo using the following primer set:

HBV-F: 5'- GGC TCT AGA GAT CCT TCG CGG GAC GTC -3' (SEQ ID NO:22) and

HBV-R: 5'- GGC GAA TTC ACT GCC TTC CAC CAA GC -3' (SEQ ID NO:23).

[0118] Amplification was done with Elongase enzyme (LTI) according to the manufacturer's instructions with the following conditions: 30 seconds at 94°C, then 5 cycles of 45 seconds at 94 °C, 1 minute at 42 °C and 1 minute at 68 °C, followed by 30 cycles of 45 seconds at 94 °C, 1 minute at 65 °C and 1 minute at 68 °C, followed by 10 minutes at 68 °C. The 625 bp PCR fragment was then purified using the Qiaquick PCR purification kit, digested with EcoRI and XbaI and purified from gel using the GENECLAN kit. The three isolated fragments were ligated and transformed into DH5 $\alpha$  competent cells (LTI) to give the construct pRSVhbvNeo (FIG. 11). In this construct, the transcription regulatory regions of the RSV expression cassette and the neomycin selection marker are modified to reduce overlap with adenoviral vectors that often contain CMV and SV40 transcription regulatory sequences.

2) Generation of Ad35 recombinant viruses on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

[0119] PER.C6 cells were seeded at a density of  $5 \times 10^6$  cells in a T25 flask and, the next day, transfected with a DNA mixture containing:

- 1  $\mu$ g pAdApt35.LacZ digested with PacI
- 5  $\mu$ g pRSV.Ad35E1 undigested
- 2  $\mu$ g pWE.Ad35.pIX-rITR digested with NotI

[0120] Transfection was done using Lipofectamine according to the manufacturer's instructions. Five hours after addition of the transfection mixture to the cells, medium was removed and replaced by fresh medium. After two days, cells were transferred to T80 flasks and further cultured. One week post-transfection, 1 ml of the medium was added to A549 cells and, the following day, cells were stained for LacZ expression. Blue cells were clearly visible after two hours of staining indicating that recombinant LacZ expressing viruses were produced. The cells were further cultured, but no clear appearance of CPE was noted. However, after 12 days, clumps of cells appeared in the monolayer and 18 days following transfection, cells were detached. Cells and medium were then harvested, freeze-thawed once, and 1 ml of the crude lysate was used to infect PER.C6 cells in a 6-well plate. Two days after infection, cells were stained for



LacZ activity. After two hours, 15% of the cells were stained blue. To test for the presence of wt and / or replicating competent viruses, A549 cells were infected with these viruses and further cultured. No signs of CPE were found indicating the absence of replication-competent viruses. These experiments show that recombinant AdApt35.LacZ viruses were made on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

[0121] Ad35 recombinant viruses escape neutralization in human serum containing neutralizing activity to Ad5 viruses.

[0122] The AdApt35.LacZ viruses were then used to investigate infection in the presence of serum that contains neutralizing activity to Ad5 viruses. Purified Ad5-based LacZ virus served as a positive control for NA. Hereto, PER.C6 cells were seeded in a 24-well plate at a density of  $2 \times 10^5$  cells/well. The next day, a human serum sample with high neutralizing activity to Ad5 was diluted in culture medium in five steps of five times dilutions. 0.5 ml of diluted serum was then mixed with  $4 \times 10^6$  virus particles AdApt5.LacZ virus in 0.5 ml medium and after 30 minutes of incubation at 37 °C, 0.5 ml of the mixture was added to PER.C6 cells in duplicate. For the AdApt35.LacZ viruses, 0.5 ml of the diluted serum samples were mixed with 0.5 ml crude lysate containing AdApt35.LacZ virus and, after incubation, 0.5 ml of this mixture was added to PER.C6 cells *in duplo*. Virus samples incubated in medium without serum were used as positive controls for infection. After two hours of infection at 37 °C, medium was added to reach a final volume of 1 ml and cells were further incubated. Two days after infection, cells were stained for LacZ activity. The results are shown in Table II. From these results, it is clear that whereas AdApt5.LacZ viruses are efficiently neutralized, AdApt35.LacZ viruses remain infectious irrespective of the presence of human serum. This proves that recombinant Ad35-based viruses escape neutralization in human sera that contain NA to Ad5-based viruses.

#### Example 6

*Generation of cell lines capable of complementing E1-deleted Ad35 viruses*

*Generation of pIG135 and pIG270*

[0123] Construct pIG.E1A.E1B (FIG. 12) contains E1 region sequences of Ad5 corresponding to nucleotides 459 to 3510 of the wt Ad5 sequence (Genbank

accession number M72360) operatively linked to the human phosphoglycerate kinase promoter ("PGK") and the Hepatitis B Virus polyA sequences. The generation of this construct is described in International Patent Application No. WO97/00326. The E1 sequences of Ad5 were replaced by corresponding sequences of Ad35 as follows. pRSV.Ad35-E1 (described in Example 5) was digested with EcoRI and Sse8387I and the 3 kb fragment corresponding to the Ad35-E1 sequences was isolated from gel. Construct pIG.E1A.E1B was digested with Sse8387I completely and partially with EcoRI. The 4.2 kb fragment corresponding to vector sequences without the Ad5-E1 region but retaining the PGK promoter were separated from other fragments on LMP agarose gel and the correct band was excised from gel. Both obtained fragments were ligated resulting in pIG.Ad35-E1.

[0124] This vector was further modified to remove the LacZ sequences present in the pUC119 vector backbone. Hereto, the vector was digested with BsaAI and BstXI and the large fragment was isolated from gel. A double stranded oligo was prepared by annealing the following two oligos:

BB1: 5'-GTG CCT AGG CCA CGG GG-3' (SEQ ID NO:24) and

BB2: 5'-GTG GCC TAG GCA C-3' (SEQ ID NO:25).

[0125] Ligation of the oligo and the vector fragment resulted in construct pIG135 (FIG. 13). Correct insertion of the oligo restores the BsaAI and BstXI sites and introduces a unique AvrII site. Next, we introduced a unique site at the 3' end of the Ad35-E1 expression cassette in pIG135. Hereto, the construct was digested with SapI and the 3' protruding ends were made blunt by treatment with T4 DNA polymerase. The thus treated linear plasmid was further digested with BsrGI and the large vector-containing fragment was isolated from gel. To restore the 3' end of the HBVpolyA sequence and to introduce a unique site, a PCR fragment was generated using the following primers:

270F: 5'- CAC CTC TGC CTA ATC ATC TC -3' (SEQ ID NO:26) and

270R: 5'- GCT CTA GAA ATT CCA CTG CCT TCC ACC -3' (SEQ ID NO:27).

[0126] The PCR was performed on pIG.Ad35.E1 DNA using Pwo polymerase (Roche) according to the manufacturer's instructions. The obtained PCR product was digested with BsrGI and dephosphorylated using Tsap enzyme (LTI), the latter to prevent

insert dimerization on the BsrGI site. The PCR fragment and the vector fragment were ligated to yield construct pIG270 (FIG. 14).

*Ad35-E1 sequences are capable of transforming rat primary cells*

[0127] Newborn WAG/RJ rats were sacrificed at 1 week of gestation and kidneys were isolated. After careful removal of the capsule, kidneys were disintegrated into a single cell suspension by multiple rounds of incubation in trypsin/EDTA (LTI) at 37 °C and collection of floating cells in cold PBS containing 1% FBS. When most of the kidney was trypsinized, all cells were re-suspended in DMEM supplemented with 10% FBS and filtered through a sterile cheesecloth. Baby Rat Kidney (BRK) cells obtained from one kidney were plated in 5 dishes (Greiner, 6 cm). When a confluency of 70-80% was reached, the cells were transfected with 1 or 5 µgr DNA/dish using the CaPO<sub>4</sub> precipitation kit (LTI) according to the manufacturer's instructions. The following constructs were used in separate transfections: pIG.E1A.E1B (expressing the Ad5-E1 region), pRSV.Ad35-E1, pIG.Ad35-E1 and pIG270 (expressing the Ad35-E1 region). Cells were incubated at 37 °C, 5% CO<sub>2</sub> until foci of transformed cells appeared. Table III shows the number of foci that resulted from several transfection experiments using circular or linear DNA. As expected, the Ad5-E1 region efficiently transformed BRK cells. Foci also appeared in the Ad35-E1 transfected cell layer although with lower efficiency. The Ad35 transformed foci appeared at a later time point: ~2 weeks post transfection compared with 7-10 days for Ad5-E1. These experiments clearly show that the E1 genes of the B group virus Ad35 are capable of transforming primary rodent cells. This proves the functionality of the Ad35-E1 expression constructs and confirms earlier findings of the transforming capacity of the B-group viruses Ad3 and Ad7 (Dijkema, 1979). To test whether the cells in the foci were really transformed, a few foci were picked and expanded. From the 7 picked foci, at least 5 turned out to grow as established cell lines.

*Generation of new packaging cells derived from primary human amniocytes*

[0128] Amniotic fluid obtained after amniocentesis was centrifuged and cells were re-suspended in AmnioMax medium (LTI) and cultured in tissue culture flasks at

37 °C and 10 % CO<sub>2</sub>. When cells were growing nicely (approximately one cell division/24 hrs.), the medium was replaced with a 1:1 mixture of AmnioMax complete medium and DMEM low glucose medium (LTI) supplemented with Glutamax I (end concentration 4mM, LTI) and glucose (end concentration 4.5 gr/L, LTI) and 10% FBS (LTI). For transfection ~ 5x10<sup>5</sup> cells were plated in 10 cm tissue culture dishes. The day after, cells were transfected with 20 µgr of circular pIG270/dish using the CaPO<sub>4</sub> transfection kit (LTI) according to manufacturer's instructions and cells were incubated overnight with the DNA precipitate. The following day, cells were washed 4 times with PBS to remove the precipitate and further incubated for over three weeks until foci of transformed cells appeared. Once a week the medium was replaced by fresh medium. Other transfection agents like, but not limited to, LipofectAmine (LTI) or PEI (Polyethylenimine, high molecular weight, water-free, Aldrich) were used. Of these three agents, PEI reached the best transfection efficiency on primary human amniocytes: ~1% blue cells 48 hrs.

*Following transfection of pAdApt35. LacZ.*

[0129] Foci are isolated as follows. The medium is removed and replaced by PBS after which foci are isolated by gently scraping the cells using a 50-200 µl Gilson pipette with a disposable filter tip. Cells contained in ~10 µml PBS were brought in a 96 well plate containing 15 µl trypsin/EDTA (LTI) and a single cell suspension was obtained by pipetting up and down and a short incubation at room temperature. After addition of 200 µl of the above described 1:1 mixture of AmnioMax complete medium and DMEM with supplements and 10% FBS, cells were further incubated. Clones that continued to grow were expanded and analysed for their ability to complement growth of E1-deleted adenoviral vectors of different sub-groups, specifically ones derived from B-group viruses, and more specifically from Ad35 or Ad11.

*Generation of new packaging cell lines from HER cells*

[0130] HER cells are isolated and cultured in DMEM medium supplemented with 10% FBS (LTI). The day before transfection, ~5x10<sup>5</sup> cells are plated in 6 cm dishes and cultured overnight at 37 °C and 10% CO<sub>2</sub>. Transfection is done using the CaPO<sub>4</sub>

precipitation kit (LTI) according to the manufacturer's instructions. Each dish is transfected with 8-10 µmgr pIG270 DNA, either as a circular plasmid or as a purified fragment. To obtain the purified fragment, pIG270 was digested with AvrII and XbaI and the 4 kb fragment corresponding to the Ad35-E1 expression cassette was isolated from gel by agarase treatment (Roche). The following day, the precipitate is washed away carefully by four washes with sterile PBS. Then fresh medium is added and transfected cells are further cultured until foci of transformed cells appear. When large enough (>100 cells), foci are picked and brought into 96-wells as described above. Clones of transformed HER cells that continue to grow,, are expanded and tested for their ability to complement growth of E1-deleted adenoviral vectors of different sub-groups, specifically ones derived from B-group viruses, and more specifically from Ad35 or Ad11.

*New packaging cell lines derived from PER.C6*

[0131] As described in Example 5, it is possible to generate and grow Ad35-E1-deleted viruses on PER.C6 cells with cotransfection of an Ad35-E1 expression construct, *e.g.*, pRSV.Ad35.E1. However, large-scale production of recombinant adenoviruses using this method is cumbersome because, for each amplification step, a transfection of the Ad35-E1 construct is needed. In addition, this method increases the risk of non-homologous recombination between the plasmid and the virus genome with high chances of generation of recombinant viruses that incorporate E1 sequences resulting in replication-competent viruses. To avoid this, the expression of Ad35-E1 proteins in PER.C6 has to be mediated by integrated copies of the expression plasmid in the genome. Since PER.C6 cells are already transformed and express Ad5-E1 proteins, addition of extra Ad35-E1 expression may be toxic for the cells. However, it is not impossible to stably transfect transformed cells with E1 proteins since Ad5-E1-expressing A549 cells have been generated.

[0132] In an attempt to generate recombinant adenoviruses derived from subgroup B virus Ad7, Abrahamsen *et al.* (1997) were not able to generate E1-deleted viruses on 293 cells without contamination of wt Ad7. Viruses that were picked after plaque purification on 293-ORF6 cells (Brough *et al.*, 1996) were shown to have incorporated Ad7-E1B sequences by nonhomologous recombination. Thus, efficient

propagation of Ad7 recombinant viruses proved possible only in the presence of Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B proteins are known to interact with cellular as well as viral proteins (Bridge *et al.*, 1993; White, 1995). Possibly, the complex formed between the E1B-55K protein and E4-ORF6 which is necessary to increase mRNA export of viral proteins and to inhibit export of most cellular mRNAs is critical and, in some way, serotype-specific. The above experiments suggest that the E1A proteins of Ad5 are capable of complementing an Ad7-E1A deletion and that Ad7-E1B expression in adenovirus packaging cells on itself is not enough to generate a stable complementing cell line. To test whether one or both of the Ad35-E1B proteins is/are the limiting factor in efficient Ad35 vector propagation on PER.C6 cells, we have generated an Ad35 adapter plasmid that does contain the E1B promoter and E1B sequences but lacks the promoter and the coding region for E1A. Hereto, the left end of wt Ad35 DNA was amplified using the primers 35F1 and 35R4 (both described in Example 4) with Pwo DNA polymerase (Roche) according to the manufacturer's instructions. The 4.6 kb PCR product was purified using the PCR purification kit (LTI) and digested with SnaBI and ApaI enzymes. The resulting 4.2 kb fragment was then purified from gel using the QIAExII kit (Qiagen). Next, pAdApt35IP1 (Example 4) was digested with SnaBI and ApaI and the 2.6 kb vector-containing fragment was isolated from gel using the GENECLAN kit (BIO 101, Inc). Both isolated fragments were ligated to give pBr/Ad35.leftITR-pIX (FIG.15). Correct amplification during PCR was verified by a functionality test as follows: The DNA was digested with BstBI to liberate the Ad35 insert from vector sequences and 4 µg of this DNA was cotransfected with 4 µg of NotI digested pWE/Ad35.pIX-rITR (Example 4) into PER.C6 cells. The transfected cells were passaged to T80 flasks at day 2 and again two days later CPE had formed showing that the new pBr/Ad35.leftITR-pIX construct contains functional E1 sequences. The pBr/Ad35.leftITR-pIX construct was then further modified as follows. The DNA was digested with SnaBI and HindIII and the 5' HindII overhang was filled in using Klenow enzyme. Religation of the digested DNA and transformation into competent cells (LTI) gave construct pBr/Ad35.leftITR-pIXΔE1A (FIG. 16). This latter construct contains the left end 4.6 kb of Ad35 except for E1A sequences between bp 450 and 1341 (numbering according to SEQ ID NO:44) and thus lacks the E1A promoter and most of the E1A

coding sequences. pBr/Ad35.leftITR-pIXΔE1A was then digested with BstBI and 2 μg of this construct was cotransfected with 6 μmgr of NotI digested pWE/Ad35.pIX-rITR (Example 4) into PER.C6 cells. One week following transfection, full CPE had formed in the transfected flasks.

[0133] This experiment shows that the Ad35-E1A proteins are functionally complemented by Ad5-E1A expression in PER.C6 cells and that at least one of the Ad35-E1B proteins cannot be complemented by Ad5-E1 expression in PER.C6. It further shows that it is possible to make a complementing cell line for Ad35-E1-deleted viruses by expressing Ad35-E1B proteins in PER.C6. Stable expression of Ad35-E1B sequences from integrated copies in the genome of PER.C6 cells may be driven by the E1B promoter and terminated by a heterologous poly-adenylation signal like, but not limited to, the HBVpA. The heterologous pA signal is necessary to avoid overlap between the E1B insert and the recombinant vector, since the natural E1B termination is located in the pIX transcription unit that has to be present on the adenoviral vector. Alternatively, the E1B sequences may be driven by a heterologous promoter like, but not limited to, the human PGK promoter or by an inducible promoter like, but not limited to, the 7xtetO promoter (Gossen and Bujard, 1992). Also, in these cases, the transcription termination is mediated by a heterologous pA sequence, *e.g.*, the HBV pA. The Ad35-E1B sequences at least comprise one of the coding regions of the E1B-21K and the E1B-55K proteins located between nucleotides 1611 and 3400 of the wt Ad35 sequence. The insert may also include part of the Ad35-E1B sequences between nucleotides 1550 and 1611 of the wt Ad35 sequence (SEQ ID NO:44).

#### Example 7

*Ad35-based viruses deleted for E1A and E1B-21K genes efficiently propagate on Ad5 complementing cell lines.*

[0134] The generation of Ad35-based viruses that are deleted for E1A and retain the full E1B region is described in Example 6 of this application. Such viruses can be generated and propagated on the Ad5 complementing cell line PER.C6. The E1B region comprises partially overlapping coding sequences for the two major proteins 21K and 55K (Bos *et al.*, 1981). Whereas, during productive wt adenoviral infection, both

21K and 55K are involved in counteracting the apoptose-inducing effects of E1A proteins, the E1B-55K protein has been suggested to have additional functions during the late phase of virus infection. These include the accumulation of viral mRNAs, the control of late viral gene expression and the shutoff of most host mRNAs at the level of mRNA transport (Babiss *et al.*, 1984, 1985; Pilder *et al.*, 1986). A complex formed between E1B-55K and the ORF6 protein encoded by the adenovirus early region 4 (Leppard and Shenk, 1989; Bridge and Ketner, 1990) exerts at least part of these functions.

[0135] To analyze which of the E1B proteins is required for propagation of Ad35-E1A-deleted recombinant viruses on PER.C6 packaging cells, the E1B region in construct pBr.Ad35.leftITR-pIXΔE1A (see Example 6 and FIG. 16) was further deleted. A first construct, pBr.Ad35Δ21K, retains the full E1B-55K sequence and is deleted for E1A and E1B-21K. Hereto, pBr.Ad35.leftITR-pIXΔE1A was digested with NcoI and BspEI and the 5 KB vector fragment was isolated from agarose gel using the GENECLAN kit (BIO 101, Inc.) according to the manufacturer's instructions. Then a PCR fragment was generated with pBr.Ad35.leftITR-pIXΔE1A as template DNA using the following primers:

35D21: 5'- TTA GAT CCA TGG ATC CCG CAG ACT C-3' (SEQ ID NO:28) and

35B3: 5'- CCT CAG CCC CAT TTC CAG-3' (SEQ ID NO:29).

Amplification was done using Pwo DNA polymerase (Roche) according to manufacturer's recommendations with the addition of DMSO (final concentration 3%) in the reaction mixture. The PCR program was as follows: 94°C for 2', then 30 cycles of 94°C for 30'', 58°C for 30'' and 72°C for 45'' and a final step at 68°C for 8' to ensure blunt ends.

[0136] This PCR amplifies Ad35-E1B sequences from nucl. 1908 to 2528 (sequence Ad35, SEQ ID NO:44) and introduces an NcoI site at the start codon of the E1B-55K coding sequence (bold in primer 35D21). The 620 bp PCR fragment was purified using the PCR purification kit (Qiagen) and then digested with NcoI and BspEI, purified from agarose gel as above and ligated to the above-described NcoI/BspEI digested vector fragment to give pBr.Ad35Δ21K (FIG. 17).

[0137] Since the coding regions of the 21K and 55K proteins overlap, it is only possible to delete part of the 55K coding sequences while retaining 21K. Hereto,



pBr.Ad35.leftITR-pIXΔE1A was digested with BglII and the vector fragment was religated to give pBr.Ad35Δ55K1 (FIG. 18). This deletion removes E1B coding sequences from nucl. 2261 to 3330 (Ad35 sequence in SEQ ID NO:44). In this construct the N-terminal 115 amino acids are retained and become fused to 21 additional amino acids out of the proper reading frame before a stop codon is encountered. The 21K coding region is intact in construct pBr.Ad35Δ55K1.

[0138] A third construct that has a deletion of E1A, 21K and most of the 55K sequences was generated as follows. pBr.Ad35.leftITR-pIX (FIG. 15) was digested with SnaBI and MfeI (isoschizomer of MunI) and the 5' overhang resulting from the MfeI digestion was filled in using Klenow enzyme. The 4.4 kb vector fragment was isolated from gel using the GENECLEAN kit (Bio 101, Inc.) according to the manufacturer's instructions and religated to give construct pBr.Ad35ΔSM (FIG. 19). In this construct, the Ad35 sequences between nucl. 453 and 2804 are deleted. Thus, 596 nucl. of the 3' end of E1b-55K are retained. A further deletion of 55K sequences was made in construct pBr.Ad35ΔE1A.ΔE1B by digestion of pBr.Ad35.leftITR-pIX with SnaBI and BglII, Klenow treatment to fill in the BglII cohesive ends, and religation. FIG. 20 shows a schematic representation of the above-mentioned constructs.

[0139] To test whether Ad35-based viruses can be generated with these constructs, each of the constructs was cotransfected with NotI digested pWE.Ad35pIX-rITR (see, Example 4) onto PER.C6 cells. Hereto, the respective fragments were PCR amplified using primers 35F1 and 35R4 (see, Example 4). This PCR amplification was done since some of the constructs were difficult to isolate in large enough quantities. In this way, equal quality of the different adapter fragments was ensured. For the amplification, Pwo DNA polymerase (Roche) was used according to the manufacturer's instructions but with DMSO (3% final concentration) added to the PCR mixture. Of each template ~ 50 ng DNA was used. The conditions for the PCR were as follows: 94°C for 2', then 5 cycles of 94°C for 30'', 48°C for 45'' and 72°C for 4' followed by 25 cycles of 94°C for 30'', 60°C for 30'' and 72°C for 4' and a final step at 68°C for 8'.

[0140] PCR fragments were generated from pBr.Ad35leftITR-pIX, pBr.Ad35.leftITR-pIXΔE1A, pBr.Ad35Δ21K, pBr.Ad35Δ55K1,

pBr.Ad35 $\Delta$ SM and pBr.Ad35 $\Delta$ E1A $\Delta$ E1B. All fragments were using the PCR purification kit (Qiagen) according to manufacturer's instructions and final concentrations were estimated on EtBr stained agarose gel using the Eagle Eye II Still Video system and EagleSight software (Stratagene) with the SmartLadder molecular weight marker (Eurogentec) as reference.

[0141] PER.C6 cells were seeded at a density of  $2.5 \times 10^6$  cells in a T25 culturing flask in DMEM containing 10% fetal calf serum (FCS) and 10mM MgSO<sub>4</sub> and cultured in a humidified stove at 37°C, 10% CO<sub>2</sub>. The next day, 3 mg of each of the PCR fragments was cotransfected with 5  $\mu$ gr NotI digested pWE.Ad35pIX-rITR using LipofectAmine (GIBCO, Life Technologies Inc.) according to the manufacturer's instructions. Two days after the transfection, all cells were passed to a T80 flask and further cultured. Cultures were then monitored for the appearance of CPE. In line with the outcome of previous experiments described in Examples 4 and 6, pBr.Ad35.leftITR-pIX and pBr.Ad35.leftITR-pIX $\Delta$ E1A showed almost full CPE within one week following transfection. Of the fragments with different E1B deletions, only pBr.Ad35 $\Delta$ 21K showed CPE at the same time as the above two fragments. Constructs pBr.Ad35 $\Delta$ 55K1, pBr.Ad35 $\Delta$ SM and pBr.Ad35 $\Delta$ E1A $\Delta$ E1B did not give CPE at all, not even after harvesting by freeze-thawing and re-infection of the crude lysate onto fresh PER.C6 cells.

[0142] From these experiments, it can be concluded that Ad35-E1B-55K, and not E1B-21K, is necessary for generation and propagation of Ad35-based viruses on Ad5 complementing cell lines. Therefore, Ad35-based viruses having a deletion of the E1A and E1B-21K genes and having the E1B-55K gene or a functional fragment thereof, can be grown on Ad5 complementing cell lines. Alternatively, Ad35-based viruses can be grown on PER.C6 cells that stably express the full E1B region or the E1B-55K gene, or a functional fragment thereof. The Ad35-E1B-55K gene, or functional parts thereof, may be expressed from a heterologous promoter like, but not limited to, the human PGK promoter, the human cytomegalovirus immediate early promoter (CMV), Rous sarcoma virus promoter, etc., and terminated by a heterologous poly adenylation sequence (pA) like, but not limited to, the hepatitis B virus poly adenylation sequence (HBVpA) and the

Simian Virus 40 poly adenylation sequence (SV40pA), etc. As nonlimiting examples, PER.C6 cells that express the Ad35-E1B region driven by the E1B promoter and HBVpA, PER.C6 cells that express the Ad35-E1B region driven by the human PGK promoter and HBVpA and PER.C6 cells that express a functional fragment of Ad35-E1B-55K driven by the human PGK promoter and HBVpA are described below.

*Generation of pIG35BL and pIG35BS*

[0143] We describe the generation of two expression constructs, pIG.35BS and pIG.35BL, that both carry the Ad35-E1B genes and a neomycin selection marker. The two constructs differ in the length of the fragment containing the E1B promoter. In 35BL, the promoter fragment is longer and includes the 3' end of the E1A region (103 nucl. coding sequence and pA). The E1B region is terminated by the HBVpolyA and the neo<sup>r</sup> gene is driven by a hPGK promoter/HBVpA cassette.

[0144] pIG.35BL was made as follows. Construct pRSV.Ad35E1 (described in Example 5, FIG. 8) was digested with NruI and HindIII and the protruding ends were filled in by Klenow treatment. The 7 kb vector fragment was separated from the smaller fragment on gel and isolated using the GENECLEAN kit (BIO 101, Inc.). After religation of the DNA and transformation into competent STBL2 cells (Gibco, LTI), correct clones were isolated. pIG.35BL (FIG. 21) contains 273 nucl. upstream of the start site of the E1B-21K coding region.

[0145] pIG.35BS was made in the same way as pIG.35BL except that pRSV.Ad35E1 was digested with NruI and HpaI (both enzymes leave blunt ends), resulting in a shorter fragment upstream of the coding region of E1B-21K: 97 nucleotides.

[0146] To generate Ad35-E1B expressing cells, PER.C6 cells were seeded in 10 cm dishes at  $1 \times 10^6$  cells/dish. Two days later, cells were transfected with Scal linearised constructs. Four dishes were transfected with 1 and four with 2  $\mu$ g DNA (total of 16 dishes; Lipofectamine (Gibco, LTI), no carrier DNA used) according to the manufacturer's instructions. The next day, transfected cells received G418-containing medium (0.75 mg/ml). Control transfections using LacZ expression constructs (2  $\mu$ g) were stained after 48 hrs and showed a transfection efficiency of ~25%. Four days

following addition of selection medium, untransfected cells started to die and again, three days later, clones were becoming visible. A week later, the first clones were picked. Transfection with 1  $\mu$ g resulted in less and also, initially, smaller clones (total ~20 clones/dish against >50 clones/dish for the transfection with 2  $\mu$ g DNA). The positive control transfection using 2  $\mu$ g pcDNA3 (Invitrogen) resulted in ~ 50 clones.

[0147] In total, 120 clones were picked and 107 were successfully established (55 from pIG35BS and 52 from pIG35BL).

#### *Generation of pIG35Bneo*

[0148] pIG35Bneo is an Ad35-E1B expression plasmid from which the E1B genes are expressed from a heterologous promoter (hPGK) and that also contains a neomycin resistance expression cassette. To avoid instability of the plasmid due to recombination events on homologous sequences, the RSV promoter drives the neo<sup>r</sup> gene. To achieve this, construct pRSVhbv.Neo (described in Example 5, FIG. 11) was digested with ScaI and BamHI and protruding ends were filled in using Klenow enzyme. The 1070 bp fragment containing part of the Ampicilin gene and the RSV promoter was isolated from gel using the GENECLAN kit (BIO 101, Inc.). Next, pRSVhbvNeo was digested with ScaI and EcoRI, blunted with Klenow and the 3.2 kb fragment containing the neo gene, HBVpA, vector and part of the Ampicilin gene was isolated as above. The two fragments were then ligated to give pRSVneo4 (FIG. 22). Construct pIG270 (FIG. 14, described in Example 6) was then digested with EcoRI and NcoI and sticky ends were blunted with Klenow enzyme. The vector-containing fragment was isolated from gel as described above and religated to give pIG270delE1A. This construct was digested with AvrII and XbaI and protruding ends were filled in using Klenow enzyme. The 2.9 kb fragment containing the hPGK promoter and Ad35-E1B sequences was isolated from gel as above. Next, pRSVneo4 was digested with BglII, blunted with Klenow enzyme, dephosphorylated and isolated from gel. The blunted AvrII/XbaI Ad35-E1B fragment was then ligated with the above prepared pRSVneo4 vector fragment and resulting clones were analysed. One clone that contained both expression cassettes in the same orientation was chosen and named pIG35Bneo (FIG. 23). Detailed analysis of this clone

revealed that an extra BglII site was present, probably due to an incomplete Klenow reaction (BglII site at nucl 2949 in FIG. 23).

#### *Generation of pIG35.55K*

[0149] Construct pIG35.55K is similar to pIG35Bneo, however, it lacks the coding region of Ad35-E1B-21K. Hereto, both the E1A and E1B-21K sequences are first deleted from pIG270 as follows:

[0150] Construct pIG270 is digested with EcoRI, treated with Klenow enzyme and purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. The recovered DNA is then digested with AgeI and the ~5 kb vector fragment was isolated from gel as above. Next, Ad35-E1B-55K sequences are amplified by PCR on pIG270 template DNA using the following primers:

35D21: 5'- TTA GAT CCA TGG ATC CCG CAG ACT C-3' (SEQ ID NO:28) and

35B3: 5'- CCT CAG CCC CAT TTC CAG-3' (SEQ ID NO:29)

The conditions used for the amplification are as previously described. The PCR fragment is purified using the PCR purification kit (Qiagen) and digested with NcoI. Following Klenow treatment to fill in the protruding ends, the DNA is further digested with AgeI and again column purified. The thus treated PCR fragment is then cloned into the above prepared EcoRI/AgeI digested vector fragment to give pIG270.ΔE1AΔ21K. The last steps to obtain pIG35.55K (FIG. 24) are equivalent to the last steps described above for the generation of pIG35Bneo, starting with pIG270.ΔE1AΔ21K instead of pIG270.ΔE1A.

[0151] pIG35.55K is then linearized with ScaI and used to transfect PER.C6 cells as described above. Clones that are resistant to G418 selection are picked and analysed for their ability to complement the propagation of E1-deleted Ad35 viruses.

#### **Example 8**

*New packaging cell lines for the generation and propagation of E1-deleted Ad35-based vectors derived from primary human cells.*

[0152] The complete morphological transformation of primary cells by adenovirus E1 genes is the result of the combined activities of the proteins encoded by

the E1A and E1B regions. The roles of the different E1 proteins in lytic infection and in transformation have been studied extensively (reviewed in Zantema and van der Eb, 1995; White, 1995, 1996). The adenovirus E1A proteins are essential for transformation of primary cells. The E1A proteins exert this effect through direct interaction with a number of cellular proteins that are involved in regulation of transcription. These include the pRB family of proteins, p300/CBP and TATA binding protein. In addition to this, E1A increases the level of p53 protein in the cells. In the absence of adenovirus E1B activity, the rise in p53 levels leads to the induction of apoptosis. Both proteins encoded by the E1B region counteract the induction of apoptosis, although by different mechanisms. E1B-21K seems to counteract apoptosis in a manner similar to Bcl-2 via interaction with the effector proteins downstream in the apoptosis pathway (Han *et al.*, 1996), whereas E1B-55K functions through direct interaction with p53. Importantly, the molecular mechanism by which the E1B-55K proteins of Ad2 and 5 (subgroup C) and Ad12 (subgroup A) function in the ability to neutralise p53 may differ. Whereas Ad5 E1B-55K binds p53 strongly and the complex localises to the cytoplasm, Ad12-E1B-55K binds p53 weakly and both proteins are localised in the nucleus (Zantema *et al.*, 1985; Grand *et al.*, 1999). Both proteins, however, inhibit the transactivation of other genes by p53 (Yew and Berk, 1992).

[0153] In rodent cells, the activity of E1A, together with either E1B-21K or 55K, is sufficient for full transformation, although expression of both E1B proteins together is twice as efficient (Rao *et al.*, 1992; ). In human cells, however, the activity of the E1B-55K protein seems to be more important, given the observation that E1B-55K is indispensable for the establishment of transformed cells (Gallimore, 1986).

[0154] Example 6 hereof describes the generation of pIG270. In this construct, the Ad35-E1 genes are expressed from the hPGK promoter and transcription is terminated by the HBVpA. The hPGK promoter constitutes a HincII-EcoRI fragment of the promoter sequence described by Singer-Sam *et al.* (1984). The HBVpA is located in a BamHI-BglII fragment of the Hepatitis B virus genome (Simonsen and Levinson, 1983; *see also* Genbank HBV-AF090841). As mentioned before, the promoter and polyadenylation sequences of the E1 expression constructs described in this invention

may be derived from other sources without departing from the invention. Also, other functional fragments of the hPGK and HBVpA sequences mentioned above may be used.

[0155] The functionality of pIG270 was shown by transformation of primary Baby Rat Kidney cells (BRK). Comparison with an equivalent Ad5-E1 expression construct taught that Ad35-E1 genes were less efficient in transforming these cells. The same has been found for the E1 genes of Ad12 (Bernards *et al.*, 1982).

[0156] It is unclear which E1 protein(s) determine(s) the difference in transformation efficiency of E1 sequences observed for adenoviruses from different subgroups. In the case of Ad12, transfection studies with chimeric E1A/E1B genes suggested that the efficiency of transformation of BRK cells was determined by the E1A proteins (Bernards *et al.*, 1982). The E1B-55K protein is shown *infra* to contain serotype-specific functions necessary for complementation of E1-deleted adenoviruses. If these functions are related to the regulation of mRNA distribution or another late viral function, it is unlikely that these are involved in the transformation efficiency.

[0157] Analysis of functional domains in the Ad2 or Ad5-E1B-55K proteins using insertion mutants have revealed that functions related to viral replication, late protein synthesis and host protein shut-off are not confined to specific domains but are distributed along the protein (Yew *et al.*, 1990). Using the same set of mutants, the domains important for interaction with p53 and E4-Orf6 were found to be more restricted. In addition to one common binding region (amino acids 262 to 326), p53 binding was affected by mutations at aa 180 and E4-Orf6 binding was affected by mutations at aa 143 (Yew and Berk, 1992; Rubenwolf *et al.*, 1997).

[0158] Altogether, these results indicate that it is difficult to separate the E1B-55K functions related to transformation (p53 binding) and late protein synthesis (Orf6 binding).

[0159] The invention discloses new E1 constructs that combine the high efficiency of transformation of one serotype with the serotype-specific complementation function of another serotype. These new constructs are used to transform primary human embryonic retinoblast cells and human amniocytes.

*The generation of pIG535, pIG635 and pIG735*

[0160] Construct pIG535 contains the Ad5-E1A region and E1B promoter sequences linked to the Ad35-E1B sequences. Hereto, pIG270 (FIG. 14; see example 6) was digested with EcoRI and NcoI. The 5.3 kb vector fragment was then isolated from gel using the GENECLEAN kit (BIO Inc. 101) according to the instructions of the manufacturer. Next, construct pIG.E1A.E1B (FIG. 12; see example 6) was digested with EcoRI and XbaI and the resulting 890 bp fragment was isolated as above. A third fragment was generated by PCR amplification on pIG.E1A.E1B using the following primers:

5E1A-F: 5'- GAG ACG CCC GAC ATC ACC TG -3' (SEQ ID NO:30) and

5E1B-R: 5'- CAA GCC TCC ATG GGG TCA GAT GTA AC -3' (SEQ ID NO:31).

The following PCR program was used: 94°C for 2' followed by 30 cycles of 94°C for 30'', 60 °C for 30'' and 72 °C for 1', and a final step at 72°C for 10' to ensure blunt ends.

[0161] The resulting 400 bp PCR fragment was digested with XbaI and NcoI. After gel isolation as above, the three fragments were ligated and transformed into STBL-2 bacteria. One colony containing all three fragments in the correct order was selected and designated pIG535 (FIG. 25).

[0162] Construct pIG635 contains the Ad5-E1A and a chimeric Ad5-Ad35-E1B region such that the 21K sequence is essentially from Ad5 and linked to the Ad35-E1B-55K sequences as far as not overlapping with the 21K sequences. First, part of the Ad5-E1 sequences are amplified by PCR using pIG.E1A.E1B as template and the following primers:

5AK: 5'- GAG CGA AGA AAC CCA TCT GAG -3' (SEQ ID NO:32) and

2155R: 5'- GGT CCA GGC CGG CTC TCG G -3' (SEQ ID NO:33). Amplification is accomplished with Pwo DNA polymerase (Roche) according to manufacturer's instructions. The 210 bp fragments is then purified from the primer sequences using the PCR purification kit (Qiagen).

[0163] A second PCR fragment is amplified from pIG270 DNA as described above but with the following primers:

2155F: 5'- CCG AGA GCC GGC CTG GAC -3' (SEQ ID NO:34) and



35F10: 5'- GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG -3'  
(SEQ ID NO:35).

[0164] The 1.3 kb amplified fragment is purified as above and mixed in a 1:1 molar ratio with the first PCR fragment. The mixture is then first subjected to a PCR reaction without the addition of primers using Pwo DNA polymerase and the following program: 94 °C for 2' and then 5 cycles of 94°C for 30'', 60 °C for 30'', 72 °C for 90''. Subsequently, primers 5AK and 35F10 are added at 0.6 µM concentration after which a last PCR amplifies a 1.5 kb fragment. Hereto, temperature was set as follows: 94 °C for 2', then 30 cycles of 94 °C for 30'', 60°C for 30'' and 72 °C for 90'', followed by a final step at 72°C for 10' to ensure blunt ends. The resulting product is purified using the PCR purification kit (Qiagen) as above and digested with KpnI and SbfI (isoschizomer of Sse8387I). The digested DNA is then isolated from gel using the GENECLEAN kit (BIO Inc., 101). Construct pIG.E1A.E1B is digested with KpnI and SbfI and the vector-containing fragment is isolated from gel as above. This fragment is ligated to the above prepared final PCR product and the ligation mixture is transformed into STBL-2 cells (Gibco, LTI) according to manufacturer's instructions. This gives construct pIG635 (Fig. 26).

[0165] In construct pIG735, the border between Ad5 derived sequences and Ad35 derived sequences is located more 3' than in construct pIG635. First, a BspEI site is introduced in the Ad5 sequence of construct pIG.E1A.E1B without changing the amino acid sequence. Hereto, Ad5 sequences from pIG.E1A.E1B are amplified using the following PCR primers:

[0166] 5AK: see above (SEQ ID NO:32), and Bsp-R: 5'- GCT CTA GAC CTG CAG GGT AGC AAC AAT TCC GGA TAT TTA CAA G -3' (SEQ ID NO:36). Amplification is accomplished using Pwo DNA polymerase (Roche) according to the manufacturer's instruction. The following PCR program is used: 94°C for 2' followed by 30 cycles of 94°C for 30'', 60 °C for 30'' and 72 °C for 30'', and a final step at 72°C for 10' to ensure blunt ends. The resulting 0.6 kb fragment is purified as above and digested with KpnI and SbfI and ligated to the above described KpnI/SbfI digested pIG.E1A.E1B vector fragment. Selection of colonies after transformation of STBL-2 bacteria (Life Techn. Inc.) gives construct pIG.E1Δ55K. pIG.E1Δ55K is then digested with SbfI

and partially with BspEI. The 6.4 kb SbfI-partial BspEI digested vector fragment is then isolated from gel using the geneClean kit (BIO 101, Inc.). Next, pIG270 is digested with BspEI and SbfI and the resulting 915 bp fragment is isolated from gel as above. This fragment is then ligated to the above prepared SbfI/partial BspEI digested pIG.E1Δ55K vector fragment and transformed into STBL-2 competent cells. This gives construct pIG735 (FIG. 27). Clones are analysed by restriction enzyme digestion and sequencing to ensure correct ligation of the fragments. Constructs pIG535, pIG635 and pIG735 can be used to generate complementing cell lines from primary human cells as described in Example 6.

### Example 9

#### *PER.C6-based complementing cell lines for E1-deleted Ad35 viruses.*

[0167] PER.C6 cells were seeded in 10 cm culture dishes at a density of  $3 \times 10^6$  cells/dish in DMEM (Gibco BRL) complemented with FBS (Gibco BRL) up to 10% and 10mM  $MgCl_2$  (4.9 M stock solution, Sigma). Two days later, 9 dishes were transfected with 1  $\mu$ g ScaI linearised pIG35.55K DNA (see example 7) and 9 dishes were transfected with 1.5  $\mu$ g ScaI linearised pIG35.55K DNA. Separate control dishes were transfected with 1 or 1.5  $\mu$ g ScaI linearised pAdApt35.LacZ to monitor transfection efficiency and with 1 or 1.5  $\mu$ g ScaI linearised pcDNA.nlsLacZ. pcDNA.nlsLacZ is a pcDNA3-based plasmid (Invitrogen) with the nlsLacZ gene (Bonnerot et al., 1987) driven by the CMV promoter. pcDNA.nlsLacZ also contains a  $neo^r$  expression cassette. As a negative control one extra dish was transfected with linearised pAdApt35.LacZ, a construct that lacks the  $neo^r$  selection gene. All transfections were performed with the LipofectAmine transfection kit (Invitrogen/Life Technologies) according to manufacturers instructions using 5 ml LipofectAmine reagent/ $\mu$ g DNA. Cells were incubated for 4 hrs with the transfection mixture after which the medium was replaced with PER.C6 culture medium. The next day medium was replaced with culture medium containing 0.5 mg/ml G418 (Gibco BRL) except in the two dishes that were transfected with 1 or 1.5  $\mu$ g pAdApt35.LacZ. These latter dishes were used to monitor LacZ expression two days following transfection. After X-gal staining of these cultures transfection efficiency was estimated at approximately 40% with slightly more blue cells

in the dish transfected with 1.5  $\mu$ g DNA. Selection medium was refreshed twice weekly in the remaining transfected dishes. Within two weeks following first addition of selection medium most cells in the negative control dish (transfected with 1.5  $\mu$ g pAdApt35.LacZ) were dead. In the dishes transfected with pcDNA.nlsLacZ cell clones were becoming visible. Since the cells transfected with pIG35.55K seemed to be more resistant to G418, the concentration was raised to 0.75 mg/ml 3 weeks following transfection. Three days and seven days later a total of 196 cell clones were picked from the dishes transfected with pIG35.55K and seeded in separate wells of 96-well plates.

[0168] Cells remaining after colony picking of two 10 cm dishes of the transfection with 1  $\mu$ g pIG35.55K DNA were trypsinised, pooled and expanded to give pool PER55K(1.0) The same was done for two dishes of the 1.5  $\mu$ g transfection. The PER55K(1.0) cell pool was expanded and seeded in 4 T25 flasks at a density of  $3.5 \times 10^6$  cells/flask for transfection to test virus generation. In addition, 3 T25 flasks with parental PER.C6 cells were seeded at the same density. pAdApt35.eGFP (an adapter plasmid containing the green fluorescent protein as marker gene; see example 4) was digested with PacI to liberate the adenoviral sequences from the plasmid backbone. pWE.Ad35.pIX-rITR (see, example 4) was digested with NotI to liberate the adenoviral sequences from the cosmid backbone. 2 flasks with PER.C6 cells and 2 flasks with PER55K(1.0) cells were transfected with 2  $\mu$ g digested pAdApt35.eGFP and 6  $\mu$ g digested pWE.Ad35.pIX-rITR each. One flask of each cell line was transfected with 8  $\mu$ g pAdApt35.LacZ to monitor transfection efficiency. The remaining flask with PER55K(1.0) cells served as a negative control and was treated as the others but did not receive the transfection mixture. All transfections were performed with LipofectAmine (Invitrogen/Life Techn.) according to manufacturers instructions using for each transfection a total of 8  $\mu$ g DNA and 40  $\mu$ l LipofectAmine reagent. The transfection mixture was removed after 4 hrs incubation and fresh culture medium was added. Transfections were done the day after seeding of the cells and again two days later cells in the T25 flasks were transferred to a T80 flask except for the LacZ control transfections. These were stained with X-gal solution after mild fixation. After five hours incubation with staining solution, the percentage of blue cells was estimated at approximately 90% in both flasks showing that transfection went well for both cell lines.

Four days following the passage to the T80 flasks the transfected PER55K(1.0) cultures showed starting CPE (cytopathogenic effect, indicative of virus replication) with approximately 100 events/flask. The untransfected PER55K(1.0) cells were grown confluent with no evidence of CPE. In the transfected PER.C6 cultures only three CPE events were visible in the confluent monolayer of cells. Again three days later, the transfected PER55K(1.0) cultures showed full CPE, with all cells rounded and detached in clumps. In contrast, in the PER.C6 cultures the few events of CPE had not progressed and cells were still in monolayer. This confirms earlier observations that generation of E1-deleted Ad35-based viruses on PER.C6 is very inefficient. Also the untransfected PER55K(1.0) cultures showed, as expected, a confluent monolayer with no CPE. The cells and medium in the PER55K(1.0) flasks with full CPE were harvested and subjected to two freeze/thaw cycles after which the cell debris was removed by centrifugation at 3000 rpm for 10 minutes in a table centrifuge. One of the resulting crude lysates was used to infect a fresh culture of PER55K(1.0) cells in a T175 flask (1.5 ml/flask). Cells and medium were harvested at full CPE four days later. This shows that infectious virus had formed in the initial transfections. GFP expression was confirmed by fluorescent microscopy of A549 cells infected with the crude lysate. The crude lysate was then used to analyse complementation of this E1-deleted Ad35.AdApt.eGFP virus in the individual clones as described below.

[0169] The above-described clones that were picked from the pIG35.55K transfected PER.C6 cells were expanded and were functionally tested for the ability to sustain replication of Ad35.AdApt.eGFP. Hereto, the clones were seeded at two densities in 6-well plates and one day later infected with 15 ml of the above described crude lysate. CPE was monitored the day after. Of the 146 clones tested in this way 19 gave full CPE at day 2 or 3 and 68 gave full CPE at day 5 or 6. The remaining clones had only partial CPE or showed a few non-progressing events. The latter were indistinguishable from PER.C6 cells that were taken along as a negative control.

[0170] Based on these results a selection of 24 clones was made that were further screened for the ability to generate recombinant E1-deleted viruses following transfection of the pAdApt35.GFP adapter plasmid and the large pWE.Ad35.pIX-rITR cosmid clone. Hereto, clones were plated in T25 flasks and transfected with 2 µg of the

adapter and 6 µg of the backbone plasmid using LipofectAmine as described above. Two days following the transfection, cells were transferred to T80 flasks to prevent overconfluency of the cultures. Of the 24 clones 5 gave full CPE three days after passage to T80 and another 13 clones gave progressing to full CPE the day after. The remaining 6 clones showed no CPE or only starting. In comparison: routine generation of E1-deleted Ad5 vectors on PER.C6 cells generally results in full CPE four to six days after transfer to T80 flasks.

[0171] This shows that the new clones efficiently complement E1-deleted adenovirus vectors. One of the clones (clone #16) described above was used to generate and produce multiple batches of E1 and E1/E3 deleted Ad35 viruses containing different transgenes. Hereto, virus in crude lysates resulting from transfections as described above, but using different adapter plasmids, were plaque purified on the new cell line. Single plaques were tested for transgene activity and then amplified for medium scale production in 4-8 triple layer flasks (3x175 cm/flask). Cells were harvested at full CPE and the virus was released and purified as routinely done for adenoviruses and described in example 1. The extraction step with freon to remove cellular debris was, however, replaced by a centrifugation step. Thus after incubation with DnaseI, the cell debris was centrifugated in conical 50 ml tubes (Greiner) at 8000 rpm in a table top centrifuge (Beckman Coulter Allegra 21R with fixed angle rotor) for 30 minutes at 4°C. This step is repeated in a fresh 50 ml tube untill the supernatant was clear (usually one time). The amount of virus particles was determined by HPLC (Shabram et al., 1997). Table IV presents the yields after downstream processing of medium scale productions of E1- and E1/E3-deleted Ad35 viruses on triple layer flasks with PER55K clone #16 cells. The amount of purified virus particles is comparable with the yields of Ad5-based vectors on PER.C6 cells.

[0172] We conclude that we have generated multiple cell lines that efficiently complement fully E1-deleted Ad35-based vectors. Thus, Ad35 E1B-55K expression in an Ad5 complementing cell line facilitates replication of Ad35 vectors.

## Example 10

### *New complementing cell lines from primary cells.*

[0173] Example 8 described the generation of construct pIG535, a hybrid Ad5E1A-Ad35 E1B expression plasmid. pCC536s and pIG536 are also hybrid Ad5-Ad35 E1 constructs but with the E1A region, E1B promoter and most of the E1B-19K gene derived from Ad5 and most of the E1B-55K gene derived from Ad35. Constructs pCC536s and pIG536 differ only in the heterologous poly adenylation sequence that terminates the E1B transcript: pIG536 has the HBV pA sequence and pCC536s has a synthetic pA sequence (SpA). The SpA sequence consists of the upstream sequence element (USE) of the human C2 complement gene (Moreira et al., 1995) and the synthetic pA sequence (SPA) described by Levitt et al., 1989.

[0174] The synthetic polyA sequence is build up using the following oligos: C2SPA-1: 5'- CCC TGC AGG GAC TTG ACT CAT GCT TGT TTC ACT TTC ACA TGG AAT TTC CCA GTT ATG AAA TTA ATA AAG -3' (SEQ ID NO:37) and C2SPA-2: 5'- GTC TAG ACA CAC AAA AAA CCA ACA CAC TAT TGC AAT GAA AAT AAA TTT CCT TTA TTA ATT TCA TAA CTG -3' (SEQ ID NO:38).

Oligonucleotides were mixed at 10µM concentration in 1x annealing buffer (10mM Tris HCl pH 7.5, 100mM NaCl, 1mM EDTA) and, using a PCR machine, the solution was heated to 94°C for 5 minutes and then cooled down to 65°C at 0.5°C / second and after incubation at 65 °C for 5 minutes further cooled down to 20°C at 0.05 °C / second. Subsequently, 10 µl 2mM dNTPs, 0.5 µl 1M MgCl<sub>2</sub> and 3 µl Klenow fragment (New England Biolabs) was added to 87 µl of the annealed sample and the mixture was incubated at room temperature for 30 minutes. 1 µl of the annealed and Klenow treated sample was then amplified using the following primers:

C2for: 5'- CGG GAT CCC CTG CAG GGA CTT GAC -3' (SEQ ID NO:39)

and

SPArev: 5'- TTG CGA CTT AAG TCT AGA CAC ACA AAA AAC C -3' (SEQ ID NO:40) using Pwo DNA polymerase (Roche) according to manufacturers instructions but with addition of DMSO (Sigma) to a final concentration of 3%. The PCR program was set at 94°C for 2 minutes, followed by 30 cycles of (94 °C for 30'', 55°C for 30'' and 72°C for 20''). Where in this document PCR programs are described ' means time in

minutes and '' means time in seconds. The amplified DNA was then purified using the QIAquick PCR purification kit (Qiagen) and digested with XbaI and SbfI. The digested product was then again purified with the PCR purification kit to remove the small digested ends. Construct pIG270 was also digested with XbaI and SbfI (isoschizomer of Sse8387I) and the resulting 5.9 kb vector containing fragment was isolated from gel using the GeneClean II kit (Bio101, Inc). The treated vector and PCR insert were then ligated to give pCC271 (FIG. 28). pCC271 thus contains the PGK promoter, the Ad35 E1 region (nucl. 468 to and including 3400 from Ad35 sequence in example 3 and SEQ ID NO:44) and the synthetic pA (SpA). The synthetic pA sequence was then also cloned into the construct pIG535 as follows.

[0175] pIG535 was digested with EcoRI, PstI and ScaI (All enzymes from New England Biolabs digested in NEB buffer 3) and the 3 kb insert corresponding to chimeric Ad5-Ad35 E1 region was purified using the GeneClean II kit (Bio 101, Inc.). Construct pCC271 was digested with EcoRI and PstI and the 3 kb vector fragment containing the SpA and PGK promoter was isolated as above. Both isolated fragments were ligated and transformed into STBL-2 competent cells (Invitrogen/LifeTechnologies) to give pCC535s (FIG. 29). pCC535s contains the same Ad5-Ad35 E1 sequences as pIG535 however, a different pA sequence.

[0176] For the construction of pCC536s, a subclone was made with the new hybrid E1B sequences. Hereto, Ad5 E1A/E1B21K sequences were amplified using the primers

5AK: 5'- GAG CGA AGA AAC CCA TCT GAG- 3' (SEQ ID NO:32) and  
2155R: 5'- GGT CCA GGC CGG CTC TCG G-3' (SEQ ID NO:33) with pIG.E1A.E1B (see, example 6 and FIG. 12) as template DNA using Pwo DNA polymerase (Roche) according to manufacturers instructions and in addition a final concentration of 3% DMSO. The program was set at: 94°C for 2' followed by 30 cycles of (94°C for 30'', 58°C for 30'' and 72°C for 30'') and ended with 68°C for 8'. This resulted in a 210 bp fragment corresponding to nucl. 2022-2233 of the Ad5 sequence. A second PCR was performed on pCC271 with primers

2155F: 5'- CCG AGA GCC GGC CTG GAC C-3' (SEQ ID NO:41) and

35F10: 5'- GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3'  
(SEQ ID NO:21).

[0177] The same PCR program was used but now with an elongation time of 90''. The resulting 1.3 kb fragment corresponds to nucl. 2112 to 3400 of the Ad35 sequence with an SbfI site at the 3'end. Note that primers 2155F (SEQ ID NO:41) and 2155R (SEQ ID NO:33) are fully complementary allowing assembly of the two fragments as follows:

[0178] Both PCR fragments were purified from gel using the Qiagen gel extraction kit. Aliquots of the purified samples were then mixed in equimolar ratio and used as template for an assembly PCR amplification with primers 5AK (SEQ ID NO:32) and 35F10 (SEQ ID NO:21) with Pwo DNA polymerase as above using the program settings:

94°C for 2', and 5 cycles of (94°C for 30'', 60°C for 30'' and 72°C for 2') followed by 25 cycles of (94°C for 30'', 58°C for 30'' and 72 °C for 90''). The resulting 1.5 kb fragment was purified from gel using the QIAquick gel extraction kit (Qiagen), ligated to the pCR-Script/Amp cloning vector (Stratagene) and transformed into DH5a competent cells (Invitrogen/Life Technologies) resulting in pCR535E1B (Figure 30). This construct was checked by restriction analysis and sequencing to confirm correct amplification of target sequences.

[0179] pCR535E1B was then digested with NotI and protruding ends were made blunt with Klenow fragment. The DNA was then purified using the QIAquick PCR purification kit (Qiagen) and eluted DNA was digested with PstI. The 1.5 kb fragment containing the chimeric E1 sequences from the pCR535E1B vector was purified from gel using the GeneClean II kit (Bio101, Inc.). This fragment was ligated to vector pCC535s digested with PvuII and PstI, and transformed into STBL-2 competent cells (Invitrogen/Life Technologies) to give pCC2155s (FIG. 31). To complete the pCC536s construct Ad5-E1 sequences were then cloned into the pCC2155s subclone. Hereto, pIG.E1A.E1B was digested with EcoRI and KpnI and the 1.6 kb fragment corresponding to Ad5 E1A and Ad5 E1B 21K (nucl. 459 to 2048 of the Ad5 sequence) was isolated from gel using the GeneClean kit. pCC2155s was digested with EcoRI and KpnI and the vector containing fragment was also gel purified. Ligation of both isolated fragments and



transformation into DH10B electrocompetent cells (Invitrogen/Life Technologies) resulted in pCC536s (Figure 32). The hybrid E1B sequences are shown in Figure 37 in more detail. FIG.37A shows an alignment of protein sequences of E1B-21K in the pCC536s construct with wild type (wt) Ad35 and Ad5 sequences. As can be seen most of the E1B-21K protein in pCC536s is derived from Ad5 except for the C-terminal 6 amino acids that are identical to Ad35 E1B-21K. FIG. 37B shows the same alignment for the E1B-55K proteins. In this case the N-terminal amino acids of pCC536s are identical to Ad5 upto aa 65. The remainder is identical to Ad35 E1B-55K. Obviously, different hybrid E1B-55K constructs can be designed using the general method outlined above without departing from the invention.

[0180] Construct pIG536 was made by replacing a fragment with the SpA in pCC536s with the corresponding fragment from pIG270 (example 6, FIG. 14) containing the HBVpA. Hereto, pIG270 was digested with BamHI and BglII and the 1.8 kb insert was isolated from gel using the GeneClean II kit (Bio 101, Inc.). pCC536s was digested with the same enzymes and the 4.8 kb vector containing fragment was purified from gel as above. Ligation of both isolated fragments and transformation into STBL-2 competent cells (Invitrogen/Life Technologies) gave construct pIG536 (FIG. 33).

[0181] The generated E1 constructs were tested in primary baby rat kidney (BRK) cells as described in example 6. The results (Table V) confirm earlier observations that Ad5-E1 genes more efficiently transform primary BRK cells than Ad35 E1 genes. The chimeric Ad5-Ad35 E1 expression constructs, pCC535s and pCC536s, produced more transformed colonies than the full Ad35 E1 constructs, pIG270 and pCC271. Furthermore, the use of a synthetic poly adenylation sequence in pCC535s resulted in slightly more foci compared to the HBVpA variant pIG535.

[0182] Human embryonic retinoblast (HER) cells were isolated from the eyes of aborted fetuses of 18 and 21 weeks of age. The eyes were brought in a 6 cm dish with PBS and cleared from outside tissue. An incision was made to reach the inner side and the gray cell layer at the inner back of the eyes containing the retinoblasts, was scraped off. This layer was transferred to a 14 ml tube in 2ml of PBS and tissue was allowed to sediment after which the PBS was removed. 2 ml trypsin (0.25%, no EDTA, GibcoBRL) was added and incubated for 5 minutes at 37°C with occasional swirling. Tissue pieces

were allowed to sediment and 1 ml trypsin with cells was transferred to a new tube. To this tube 4 ml culture medium (DMEM with 10% FCS) was added and the tube was stored on ice. The remaining tissue pieces in trypsin were brought in a 6 cm dish and cut into smaller pieces. These were, after addition of 2 ml fresh trypsin, again incubated in a 14 ml tube at 37°C with occasionally swirling. Then this mixture was added to the first isolated cells in culture medium and the total was centrifugated at 1000 rpm in a table top centrifuge. Supernatant was removed and cells were resuspended in 10 ml of culture medium. The isolated HER cells were plated in two 6 cm dishes and incubated at 37°C/10% CO<sub>2</sub>. Upon 90% confluency cultures were split 1:3 and further incubated. This procedure was repeated until enough dishes were obtained to be used for transfection and further culturing. Transfections were performed at different passage numbers using the CaPO<sub>4</sub> cotransfection kit (Invitrogen/Life Technologies) according to manufacturers instructions. For each dish (50-70% confluency) 20 µg DNA was used. Initial transfections were performed with pIG.E1A.E1B, an Ad5-E1 expression construct, and with pIG535, the hybrid Ad5-E1A/Ad35-E1B expression construct. 2-3 weeks following transfection transformed foci became visible in the pIG.E1A.E1B transfected dishes. On average 15-20 foci/dish were found in the dishes that were transfected with pIG.E1A.E1B. Over 30 clones were picked and transferred to 96-well plates. Upon confluency cells were passaged to larger culture plates or flasks and finally viable frozen in ampoules in liqN<sub>2</sub> from a T175 flask. All picked clones were established in this way. Transformed foci appeared much later in the dishes that were transfected with pIG535, the first around five weeks following transfection. On average 3-4 clones were found per dish. A total of 46 clones were picked from 7 weeks to 3 months after transfections of which 14 were viable and could be passaged multiple times. Of these, 2 clones (clone #45 and #75) were grown up to a T175 flask and viable frozen in ampoules in liqN<sub>2</sub>.

[0183] Primary HER cells were also transfected with constructs pCC535s and pCC536s. Transfection of pCC535s let to an average of 2 clones/dish and a total of 50 clones were picked. Of these picked clones 2 could be established. From the transfection with pCC536s, at least one clone could be established.

[0184] The above-described experiments show that primary HER cells can be transformed with hybrid Ad5-Ad35 E1 sequences. The efficiency of transformation was

lower than obtained with the complete Ad5 E1 region. We then tested whether the new cell lines could complement recombinant Ad35-based E1-deleted vectors. Hereto, the clone #45 that was obtained from the pIG535 transfection was seeded in T25 flasks at a density of  $7 \times 10^6$  cells/flask and infected with Ad35.AdApt.eGFP virus (see example 9) at a multiplicity of infection (moi) of 5 and 25 virus particles/cell. Full CPE was seen at days 4 and 5 for the moi 25 and 5 respectively. As a comparison parallel cultures of clone #45 cells that were infected with Ad5.AdApt.eGFP viruses gave full CPE at days 7 and 8 for moi 25 and 5 respectively. The initial infection efficiency was comparable for Ad5 and Ad35 viruses, ~80% (moi=5) and ~95% (moi=25) of the cells were infected with GFP virus one day following infection as measured by fluorescence microscopy. Cells from clone #75 were seeded in a 6-well plate at a density of  $2 \times 10^6$  cells/well and infected with Ad35.AdApt.eGFP or Ad5.AdApt.eGFP at moi 5 (VP/cell). Again initial infection efficiency was comparable for both viruses. Full CPE was observed at day 4 in case of Ad35.AdApt.eGFP infection whereas Ad5.AdApt.eGFP infected clone #75 cells gave full CPE on day 7. The difference in replication efficiency on Ad35 complementing cells between Ad35 and Ad5 recombinant vectors is even more clear when virus is generated by plasmid transfection. This is exemplified by the following transfection experiment. Clone #45 cells were seeded in T25 flasks at a density of  $3.5 \times 10^6$  cells and transfected three days later using LipofectAmine reagent (Invitrogen/Life Technologies) according to manufacturers instructions and described above. 2  $\mu$ g pAdApt35.eGFP adapter plasmid digested with PacI was cotransfected with 6  $\mu$ g pWE.Ad35.pIX-rITR or pWE.Ad35.pIX-rITRAE3 backbone cosmid digested with NotI. 2  $\mu$ g pAdApt.eGFP (Ad5 adapter plasmid, described in WO 00/70071) digested with PacI was cotransfected with 6  $\mu$ g pWE.Ad5.AflII-rITRsp (Ad5 backbone plasmid, described in WO 00/70071) also digested with PacI. One T25 was not transfected and served as a negative control. One day later transfection efficiencies were monitored by fluorescent microscopy and estimated at 10-15% in all eGFP transfections. Three days following transfection cells were transferred to T80 flasks and further incubated at 37°C/10%CO<sub>2</sub>. Again three days later CPE events were becoming visible in the cultures transfected with the pAdApt35.eGFP and the pWE.Ad35pIX-rITR+ or – E3. The transfections with the E3-deleted backbone contained more green fluorescent cells and more CPE events. The

transfection with Ad5 plasmids showed only around 20% green fluorescent cells, of which most were dying, and no CPE events. Two days later this difference had become bigger since cultures transfected with the pAdApt35.eGFP and the pWE.Ad35pIX-rITRAE3 clearly showed 80% CPE and cultures transfected with the pAdApt35.eGFP and the pWE.Ad35pIX-rITR constructs showed progressing CPE events. The Ad5 transfected culture did not show any progression. Table VI summarizes these results.

[0185] We conclude that the new complementing cell lines described above efficiently sustain replication of E1 deleted Ad35-based viruses and that the generation and replication of E1 deleted Ad5-based viruses is less efficient. Apparently, also Ad35-E1B55K proteins do not form a functional complex with Ad5-E4Orf6 proteins. Thus the serotype specificity for complementation is now also shown for recombinant Ad5 vectors on Ad35 packaging cells.

#### Example 11

##### *Generation of pWE.Ad.pIX-rITRAE3.*

[0186] The early region-3 of human adenoviruses contains multiple coding regions for proteins that interfere with the host immune response to adenoviral infection. When adenoviral vectors are used as vaccine carrier such interference is unwanted. Therefore, we constructed an Ad35 backbone cosmid lacking the E3 region.

[0187] Hereto, construct pBr.Ad35.PRn (FIG. 34; described in example 13 in publication EP 1 054 064 A1) was digested with StuI and MluI and the 17.3 kb vector fragment was purified from low melting point (LMP) gel using agarase enzyme (Roche) according to manufacturers instructions. Next, a PCR fragment was generated on pBr.Ad35.PRn using primers:

35E3for: 5'- AAT GAC TAA TGC AGG TGC GC-3' (SEQ ID NO:42) and

35E3rev: 5'- CGA CGC GTT GTA GTC GTT GAG CTT CTA G-3' (SEQ ID NO:43).

For the amplification Pwo DNA polymerase (Roche) was used according to manufacturers instructions and program set at: 94°C for 2', 30 cycles of (94°C for 30'', 58°C for 30''' and 72°C for 1') and a final incubation at 68°C for 8'. The 833 bp PCR product was purified using the QIAquick PCR purification kit (Qiagen) and digested with MluI and StuI. The digested DNA was purified from gel using the QIAquick gel

extraction kit (Qiagen). Both isolated fragments were ligated and transformed into DH5a competent cells (Invitrogen/Life Technologies) to give pBr.Ad35.PRnΔE3 (FIG. 35). The plasmid was checked by restriction analysis and sequencing of the PCR amplified insert. The E3 deletion was then cloned into the pWE.Ad35.pIX-rITR cosmid backbone. Hereto, pWE.Ad35.pIX-rITR (see example 4 and FIG. 7) was digested with PacI and the DNA was purified by precipitation with isopropanol and washing with 70% EtOH. Following resuspension in milliQ water, the DNA was digested with SmaI and the 22.8 kb vector containing fragment was purified from LMP gel using agarase enzyme as above. Construct pBr.Ad35.PRnΔE3 was digested with PacI and SmaI in the same manner and the 16.6 kb fragment was also isolated using agarase enzyme. Both isolated fragments were ligated using 0.5-0.6 μg of each fragment. Ligated fragments were then packaged using λ-phage packaging extracts (Stratagene) according to manufacturers instructions and mixed with STBL-2 cells. Bacteria were plated on LB+Amp plates and resulting colonies were analyzed for the presence of the correct construct. This gave construct pWE.Ad35.pIX-rITRΔE3 (FIG. 36). The E3 deletion extends from nucl. 27648 to 30320 of the Ad35 sequence (example 3) and thus spans a 2.6 kb region.

[0188] Cotransfection of NotI digested pWE.Ad35.pIX-rITRΔE3 and pIPsp-1 digested pAdApt35.eGFP onto PER55-clone #16 cells (see example 9) as described above gave rise to GFP expressing Ad35-based viruses. Upon isolation of viral DNA from these viruses, PCR amplification of the E3 region showed that the viruses were deleted for 2.6 kb of E3 sequences as expected.

Table I:

Serotype	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
1	597	8.66x10 <sup>10</sup>	5.00x10 <sup>7</sup>	3.2
2	574	1.04x10 <sup>12</sup>	3.66x10 <sup>11</sup>	0.4
3	131	1.19x10 <sup>11</sup>	1.28x10 <sup>7</sup>	4.0
4	260	4.84x10 <sup>11</sup>	2.50x10 <sup>8</sup>	3.3
5	533	5.40x10 <sup>11</sup>	1.12x10 <sup>10</sup>	1.7
6	477	1.05x10 <sup>12</sup>	2.14x10 <sup>10</sup>	1.7
7	328	1.68x10 <sup>12</sup>	2.73x10 <sup>9</sup>	2.4
9	379	4.99x10 <sup>11</sup>	3.75x10 <sup>7</sup>	4.1
10	387	8.32x10 <sup>12</sup>	1.12x10 <sup>9</sup>	3.9
12	305	3.64x10 <sup>11</sup>	1.46x10 <sup>7</sup>	4.4
13	231	4.37x10 <sup>12</sup>	7.31x10 <sup>8</sup>	3.8
15	443	5.33x10 <sup>12</sup>	1.25x10 <sup>9</sup>	3.6
16	312	1.75x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.5
17	478	1.39x10 <sup>12</sup>	1.45x10 <sup>9</sup>	3.0
19	430	8.44x10 <sup>11</sup>	8.55x10 <sup>7</sup>	4.0
20	156	1.41x10 <sup>11</sup>	1.68x10 <sup>7</sup>	3.9
21	437	3.21x10 <sup>11</sup>	1.12x10 <sup>8</sup>	3.5
22	365	1.43x10 <sup>12</sup>	5.59x10 <sup>7</sup>	3.4
23	132	2.33x10 <sup>11</sup>	1.57x10 <sup>7</sup>	4.2
24	405	5.12x10 <sup>12</sup>	4.27x10 <sup>8</sup>	4.1
25	405	7.24x10 <sup>11</sup>	5.59x10 <sup>7</sup>	4.1
26	356	1.13x10 <sup>12</sup>	1.12x10 <sup>8</sup>	4.0
27	342	2.00x10 <sup>12</sup>	1.28x10 <sup>8</sup>	4.2
28	347	2.77x10 <sup>12</sup>	5.00x10 <sup>7</sup>	4.7
29	386	2.78x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.1
30	409	1.33x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.4
31	303	8.48x10 <sup>10</sup>	2.19x10 <sup>7</sup>	3.6
33	302	1.02x10 <sup>12</sup>	1.12x10 <sup>7</sup>	5.0
34	425	1.08x10 <sup>12</sup>	1.63x10 <sup>11</sup>	0.8
35	446	3.26x10 <sup>12</sup>	1.25x10 <sup>11</sup>	1.4
36	325	9.26x10 <sup>12</sup>	3.62x10 <sup>9</sup>	3.4
37	257	5.86x10 <sup>12</sup>	2.8x10 <sup>9</sup>	3.3
38	337	3.61x10 <sup>12</sup>	5.59x10 <sup>7</sup>	4.8
39	241	3.34x10 <sup>11</sup>	1.17x10 <sup>7</sup>	4.5

Continued on next page.

Serotype #	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
42	370	1.95x10 <sup>12</sup>	1.12x10 <sup>8</sup>	4.2
43	284	2.42x10 <sup>12</sup>	1.81x10 <sup>8</sup>	4.1
44	295	8.45x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.6
45	283	5.20x10 <sup>11</sup>	2.99x10 <sup>7</sup>	4.2
46	282	9.73x10 <sup>12</sup>	2.50x10 <sup>8</sup>	4.6
47	271	5.69x10 <sup>11</sup>	3.42x10 <sup>7</sup>	4.2
48	264	1.68x10 <sup>12</sup>	9.56x10 <sup>8</sup>	3.3
49	332	2.20x10 <sup>12</sup>	8.55x10 <sup>7</sup>	4.4
50	459	7.38x10 <sup>12</sup>	2.80x10 <sup>9</sup>	3.4
51	450	8.41x10 <sup>11</sup>	1.88x10 <sup>8</sup>	3.7

**Legend to Table I:**

All human adenoviruses used in the neutralization experiments were produced on PER.C6 cells (Fallaux *et al.*, 1998) and purified on CsCl as described in example 1. The NaCl concentration at which the different serotypes eluted from the HPLC column is shown. Virus particles/ml (VP/ml) were calculated from an Ad5 standard. The titer in the experiment (CCID50) was determined on PER.C6 cells as described in Example 1 by titrations performed in parallel with the neutralization experiment. The CCID50 is shown for the 44 viruses used in this study and reflects the dilution of the virus needed to obtain CPE in 50% of the wells after 5 days. The ratio of VP/CCID50 is depicted in log<sub>10</sub> and is a measurement of the infectivity of the different batches on PER.C6 cells .

Table II. AdApt35.LacZ viruses escape neutralization by human serum.

Virus	Human serum dilution					
	no serum	10x	50x	250x	1250x	6250x
AdApt5.LacZ moi: 5 VP/cell	100 %	0 %	0 %	1 %	40 %	80 %
AdApt35.LacZ 250 µl crude lysate	100 %	100 %	100 %	100 %	100 %	100 %

Table III: The numbers of foci obtained with the different E1 expression constructs in BRK transformation experiments.  
Average # of foci/dish:

	Construct	1 µgr	5 µgr
Experiment 1	pIG.E1A.E1B	nd	60
	pIG.E1A.E1B	nd	35
	pRSVAd35E1	0	3
	pIG.Ad35.E1	3	7
Experiment 2	pIG.E1A.E1B	37	nd
	pIG.Ad35.E1	nd	2
Experiment 3	pIG.E1A.E1B	nd	140
	pIG.Ad35.E1	nd	20
	pIG270	nd	30

Table IV: Yields of E1- and E1/E3- deleted Ad35 viruses on clone #16 cells produced on triple layer flasks.

Virus	Scale(T175III flasks)	Total # of Virus Particles after DSP	VP/cell
Ad35.AdApt.eGFP	4	$7.5 \times 10^{11}$	2500
Ad35.ΔE3.AdApt.empty	8	$2 \times 10^{12}$	3300
Ad35.ΔE3.AdApt.LacZ	8	$3.8 \times 10^{11}$	600
Ad35.ΔE3.AdApt.MV-F	4	$8.8 \times 10^{11}$	2900
Ad35.ΔE3.AdApt.MV-H	8	$2.6 \times 10^{12}$	4250



**Table V: Transformation efficiencies on BRK cells with different Ad-E1 expression constructs.**

	Construct	Transfected DNA ( $\mu\text{g}$ )	# foci per dish
Experiment 1	pIG.E1A.E1B	5	44
	pIG270	5	0
	pCC271	5	0
	pIG535	5	1
	pCC535s	5	2.5
Experiment 2	pIG.E1A.E1B	4	15
	pCC271	4	0
	pCC535s	4	3
	pCC536s	4	3

Table VI: Generation of recombinant Ad35 viruses on the new established complementing cell line clone #45.

	GFP Expression				x
	Day1	Day3	Day 6	Day 8	
Transfected constructs					
pAdApt35.eGFP + pWE.Ad35.pIX-rITR	15%	20%	30%	50%	
pAdApt35.eGFP + pWE.Ad35.pIX-rITRΔE3	10%	25%	40-50%	100%	
pAdApt5.eGFP+ pWE.Ad5.AflII-rITR	15%	25%	20%	20%	
untransfected	0%	0%	0%	0%	

  

	CPE events				x
	Day1	Day3	Day 6	Day 8	
Transfected constructs					
pAdApt35.eGFP + pWE.Ad35.pIX-rITR	0	0	1	several	
pAdApt35.eGFP + pWE.Ad35.pIX- rITRΔE3	0	0	several	80%	
pAdApt5.eGFP+ pWE.Ad5.AflII-rITR	0	0	0	0	
untransfected	0	0	0	0	

### References:

- Abrahamsen, K., Kong, H.-L., Mastrangeli, A., Brough, D., Lizonova, A., Crystal, R.G. and Falck-Pedersen, E. (1997). Construction of an adenovirus type 7a E1A<sup>-</sup> vector. *J. Virol.* 71, 11, p8946-8951.
- Babiss, L.E. and Ginsberg, H.S. (1984). Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. *J. Virol.* 50, p202-2122.
- Babiss, L.E., Ginsberg, H.S. and Darnell, J.J. (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol. Cell. Biol.* 5, p2552-2558.
- Bernards, R., Houweling, A. Schrier, P.I., Bos, J.L. and van der Eb, A.J. (1982). Characterization of cells transformed by Ad5/Ad12 hybrid early region 1 plasmids. *Virology* 120, p422-432.
- Bonnerot, C., Rocancourt, D., Briand, P., Grimber, G. and Nicolas, JF. (1987). A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA* 84(19), p6795-6799.
- Bos, J.L., Polder, L.J., Bernards, R., Schrier, P., van den Elsen, P.J., van der Eb, A.J. and van Ormondt, H. (1981). The 2.2 kb mRNA of the E1B region of human adenovirus type 12 and 5 directs the synthesis of two major tumor antigens from different AUG triplets. *Cell* 12, p721-732.
- Bridge, E. and Ketner, G. (1990). Interaction of adenoviral E4 and E1b products in late gene expression. *Virology* 174, p345-353.
- Bridge, E., Medghalchi, S., Ubol, S., Leesong, M. and Ketner, G. (1993). Adenovirus early region 4 and viral DNA synthesis. *Virology* 193, p794-801.
- Brough, D.E., Lizonova, A., Hsu, C., Kulesa, V.A. and Kovesdi, I. (1996). A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions 1 and 4. *J. Virol.* 70, p6497-6501.
- Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., van Ormondt, H., Hoebe, R.C. and van der Eb, A.J. (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* 7 (2), p215-222.

- Fallaux, F.J., Bout, A., van der Velde, I., van den Wollenberg, D.J., Hehir, K.M., Keegan, J., Auger, C., Cramer, S.J., van Ormondt, H., van der Eb, A.J., Valerio, D. and Hoebe, R.C. (1998). New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication competent adenoviruses. *Hum. Gene Ther.* 9, 1909-1917.
- Gallimore, P.H., Grand, R.J.A. and Byrd, P.J. (1986). Transformation of human embryo retinoblasts with simian virus 40, adenovirus and ras oncogenes. *AntiCancer Res.* 6, p499-508.
- Gossen, M., and H. Bujard (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* 89; 5547-5551.
- Graham, F.O., Smiley, J., Russell, W. and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, p59-72.
- Grand, R.J.A., Parkhill, J., Szeszak, T., Rookes, S.M., Roberts, S. and Gallimore, P.H. (1999). Definition of a major p53 binding site on Ad2-E1B-58K protein and a possible nuclear localization signal on the Ad12-E1B-54K protein. *Oncogene* 18, p955-965.
- Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D. and White, E. (1996). The E1B-19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev.* 10 (4), p461-477.
- Jochemsen, A.G., Peltenburg L.T., te Pas, M.F., de Wit, C.M., Bos, J.L. and van der Eb, A.J. (1987). Activation of adenovirus 5 E1A transcription by region E1B in transformed primary rat cells. *EMBO J.* 6 (11), p3399-3405.
- Moreira, A., Wollerton, M., Monks, J. and Proudfoot, N.J. (1995). Upstream sequence elements enhance poly(A) site efficiency of the C2 complement gene and are phylogenetically conserved. *EMBO J.* 14 (15), p3809-3819.
- Leppard, K.N. and Shenk, T. (1989). The adenovirus E1B-55kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. *EMBO J.* 8, p2329-2336.
- Levitt, N., Briggs, D., Gil, A. and Proudfoot, N.J. (1989). Definition of an efficient synthetic poly(A) site. *Genes Dev.* 3, p1019-1025.

Pilder, S., Moore, M., Logan, J. and Shenk, T. (1986). The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* 6, p470-476.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S. and White, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B-19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* 89, p7742-7746.

Rubenwolf, S., Schütt, H., Nevels, M., Wolf, H. and Dobner, T. (1997). Structural analysis of the adenovirus type 5 E1B-55-kilodalton-E4orf6 protein complex. *J. Virol.* 71, p1115-1123.

Singer-Sam, J., Keith, D.H., Tani, K., Simmer, R.L., Shively, L., Lindsay, S., Yoshida, A. and Riggs, A.D. (1984). Sequence of the promoter region of the gene for human X-linked 3-phosphoglycerate kinase. *Gene* 32 (3), p409-417.

White, E. and Cipriani, R. (1990). Role of adenovirus E1B proteins in transformation: Altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. *Mol. Cell. Biol.* 10, p120-130.

White, E. (1995). Regulation of p53-dependent apoptosis by E1A and E1B. In: *The molecular repertoire of adenoviruses III*. Eds. Doerfler, W. and Böhm, P.. Springer-Verlag Berlin Heidelberg 1995, p33-58.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes Dev.* 10 (1), p1-15.

Yew, P.R., Kao, C.C. and Berk, A.J. (1990). Dissection of functional domains in the adenovirus 2 early region 1B-55K polypeptide by suppressor-linker insertional mutagenesis. *Virology* 179, p795-805.

Yew, P.R. and Berk, A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early region 1B protein. *Nature* 357, p82-85.

Simonsen, C.C. and Levinson, A.D. (1983). Analysis of processing and polyadenylation signals of the hepatitis B virus surface antigen gene by using simian virus 40-hepatitis B virus chimeric plasmids. *Mol. and Cell. Biol.* 3 (12), p2250-2258.

Zantema, A., Fransen, J.A., Davis, O.A., Ramaekers, F.C., Vooijs, G.P., DeLeys, B. and van der Eb, A.J. (1985). Localization of the E1B proteins of adenovirus 5 in

transformed cells, as revealed by interaction with monoclonal antibodies. Virology 142, p44-58.

Zanema, A. and van der Eb, A.J. (1995). Modulation of gene expression by adenovirus transformation. In: The molecular repertoire of adenoviruses III. Eds. Doerfler, W. and Böhm, P. Springer-Verlag Berlin Heidelberg 1995, p1-23.



SEQUENCE LISTING

<110> Vogels, Ronald  
Havenga, Menzo J.E.  
Mehtali, Majid

<120> Complementing cell lines

<130> P58204US10

<140> 10/002,750

<141> 2001-11-15

<150> US 09/713,678

<151> 2000-11-15

<160> 50

<170> PatentIn Ver. 2.1

<210> 1

<211> 14

<212> DNA

<213> adenoviridae

<220>

<221> misc\_feature

<222> (1)..(14)

<223> /note="5'end"

<400> 1

ccaataatat acct

14

<210> 2

<211> 21

<212> DNA

<213> adenoviridae

<220>

<221> misc\_feature

<222> (1)..(21)

<223> /note="3'end"

<400> 2

aggtatatta ttgatgatgg g

21

<210> 3

<211> 18

<212> DNA

<213> adenoviridae

<220>

<221> misc\_feature

<222> (1)..(18)

<223> /note="Terminal sequence"

<400> 3  
catcatcaat aatatacc

18

<210> 4  
<211> 47  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: oligo  
ExSalPacF

<220>  
<221> misc\_feature  
<222> (1)..(47)

<400> 4  
tcgatggcaa acagctatta tgggtattat gggttcgaat taattaa

47

<210> 5  
<211> 47  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: oligo  
ExSalPacR

<220>  
<221> misc\_feature  
<222> (1)..(47)

<400> 5  
tcgattaatt aattcgaacc cataataccc ataatagctg tttgcca

47

<210> 6  
<211> 42  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer  
PCLIPMSF

<220>  
<221> misc\_feature  
<222> (1)..(42)

<400> 6  
ccccaattgg tcgaccatca tcaataatat accttatttt gg

42

<210> 7



<211> 22  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer  
pCLIPBSRGI

<220>  
<221> misc\_feature  
<222> (1)..(22)

<400> 7  
gcgaaaattg tcacttcctg tg

22

<210> 8  
<211> 37  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: oligo  
Ecolinker+

<220>  
<221> misc\_feature  
<222> (1)..(37)

<400> 8  
aattcggcgc gccgtcgacg atatcgatag cgcgccg

37

<210> 9  
<211> 37  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: oligo  
Ecolinker-

<220>  
<221> misc\_feature  
<222> (1)..(37)

<400> 9  
aattcgggcc gctatcgata tcgtcgacgg cgcgccg

37

<210> 10  
<211> 49  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:

oligonucleotide HindXba+

<220>

<221> misc\_feature

<222> (1)..(49)

<400> 10

agctctagag gatccggttaa cgctagcgaa ttcaccggtta ccaagctta

49

<210> 11

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide HindXba-

<220>

<221> misc\_feature

<222> (1)..(49)

<400> 11

ctagtaagct tggtagcggt gaattcgcta gcgttaacgg atcctctag

49

<210> 12

<211> 44

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer 35F1

<220>

<221> misc\_feature

<222> (1)..(44)

<400> 12

cggaattctt aattaatcga catcatcaat aatatacctt atag

44

<210> 13

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer 35R2

<220>

<221> misc\_feature

<222> (1)..(33)

<400> 13

ggtaggtccta ggctgacacc tacgtaaaaa cag

33

<210> 14  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 335F3

<220>  
<221> misc\_feature  
<222> (1)..(30)

<400> 14  
tggtggagat ctggtgagta ttgggaaaac

30

<210> 15  
<211> 37  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 435R4

<220>  
<221> misc\_feature  
<222> (1)..(37)

<400> 15  
cggaattctt aattaaggga aatgcaaadc tgtgagg

37

<210> 16  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 535F5

<220>  
<221> misc\_feature  
<222> (1)..(34)

<400> 16  
cggaattcgc ggccgcggtg agtattggga aaac

34

<210> 17  
<211> 22  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 635R6

<220>  
 <221> misc\_feature  
 <222> (1)..(22)  
  
 <400> 17  
 cgccagatcg tctacagaac ag 22  
  
 <210> 18  
 <211> 23  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer 735F7  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(23)  
  
 <400> 18  
 gaatgctggc ttcagttgta atc 23  
  
 <210> 19  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer 835R8  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(42)  
  
 <400> 19  
 cggaattcgc ggccgcattt aaatcatcat caataatata cc 42  
  
 <210> 20  
 <211> 33  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer 135F11  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(33)  
  
 <400> 20  
 ggggtaccga attctcgcta ggggtatttat acc 33

<210> 21  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 235F10

<220>  
<221> misc\_feature  
<222> (1)..(38)

<400> 21  
gctctagacc tgcaggttag tcagtttctt ctccactg

38

<210> 22  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 3HBV-F

<220>  
<221> misc\_feature  
<222> (1)..(27)

<400> 22  
ggctctagag atccttcgcg ggacgtc

27

<210> 23  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 4HBV-R

<220>  
<221> misc\_feature  
<222> (1)..(26)

<400> 23  
ggcgaattca ctgccttcca ccaagc

26

<210> 24  
<211> 17  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide 1BB1

<220>  
<221> misc\_feature  
<222> (1)..(17)

<400> 24  
gtgcctaggc cacgggg

17

<210> 25  
<211> 13  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide 2BB2

<220>  
<221> misc\_feature  
<222> (1)..(13)

<400> 25  
gtggcctagg cac

13

<210> 26  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 3270F

<220>  
<221> misc\_feature  
<222> (1)..(20)

<400> 26  
cacctctgcc taatcatctc

20

<210> 27  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 4270R

<220>  
<221> misc\_feature  
<222> (1)..(27)

<400> 27  
gctctagaaa ttccactgcc ttccacc

27

<210> 28  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 135D21/  
535D21

<220>  
<221> misc\_feature  
<222> (1)..(25)

<400> 28  
ttagatccat ggatcccgca gactc

25

<210> 29  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer  
235B3/635B3

<220>  
<221> misc\_feature  
<222> (1)..(18)

<400> 29  
cctcagcccc atttccag

18

<210> 30  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 15E1A-F

<220>  
<221> misc\_feature  
<222> (1)..(20)

<400> 30  
gagacgcccg acatcacctg

20

<210> 31  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 25E1B-R

<220>  
 <221> misc\_feature  
 <222> (1)..(26)  
  
 <400> 31  
 caagcctcca tggggtcaga tgtaac 26  
  
 <210> 32  
 <211> 21  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer  
 45AK/5AK  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(21)  
  
 <400> 32  
 gagcgaagaa acccatctga g 21  
  
 <210> 33  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer  
 52155R/2155R  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(19)  
  
 <400> 33  
 ggtccaggcc ggctctcgg 19  
  
 <210> 34  
 <211> 18  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: primer 62155F  
  
 <220>  
 <221> misc\_feature  
 <222> (1)..(18)  
  
 <400> 34  
 ccgagagccg gcctggac 18



<210> 35  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer  
735F10/35F10

<220>  
<221> misc\_feature  
<222> (1)..(38)

<400> 35  
gctctagacc tgcaggtag tcagtttctt ctccactg 38

<210> 36  
<211> 43  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer Bsp-R

<220>  
<221> misc\_feature  
<222> (1)..(43)

<400> 36  
gctctagacc tgcaggtag caacaattcc ggatatttac aag 43

<210> 37  
<211> 69  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide C2SPA-1

<220>  
<221> misc\_feature  
<222> (1)..(69)

<400> 37  
ccctgcaggg acttgactca tgcttgtttc actttcacat ggaatttccc agttatgaaa 60  
ttaataaag 69

<210> 38  
<211> 69  
<212> DNA  
<213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
     oligonucleotide C2SPA-2

<220>  
 <221> misc\_feature  
 <222> (1)..(69)

<400> 38  
 gtctagacac acaaaaaaacc aacacactat tgcaatgaaa ataaatttcc ttattattaatt 60  
 tcataactg 69

<210> 39  
 <211> 24  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer C2for

<220>  
 <221> misc\_feature  
 <222> (1)..(24)

<400> 39  
 cgggatcccc tgcagggact tgac 24

<210> 40  
 <211> 31  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer SPArev

<220>  
 <221> misc\_feature  
 <222> (1)..(31)

<400> 40  
 ttgcgactta agtctagaca cacaaaaaac c 31

<210> 41  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer 2155F

<220>  
 <221> misc\_feature  
 <222> (1)..(19)

<400> 41  
ccgagagccg gcctggacc 19

<210> 42  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 35E3for

<220>  
<221> misc\_feature  
<222> (1)..(20)

<400> 42  
aatgactaat gcaggtgcgc 20

<210> 43  
<211> 28  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer 35E3rev

<220>  
<221> misc\_feature  
<222> (1)..(28)

<400> 43  
cgacgcgttg tagtcgttga gcttctag 28

<210> 44  
<211> 34794  
<212> DNA  
<213> adenoviridae

<220>  
<221> misc\_feature  
<222> (1)..(34794)  
<223> /note="Nucleic acid sequence of Ad 35"

<400> 44  
catcatcaat aatatacctt atagatggaa tgggtgccaat atgtaaatga ggtgatttta 60  
aaaagtgtgg gccgtgtggt gattggctgt ggggttaacg gttaaaaggg gcggcgcggc 120  
cgtgggaaaa tgacgtttta tgggggtgga gtttttttgc aagttgtcgc gggaaatgtt 180  
acgcataaaa aggcttcttt tctcacggaa ctacttagtt ttcccacggt atttaacagg 240  
aaatgaggta gttttgaccg gatgcaagtg aaaattgctg attttcgcgc gaaaactgaa 300  
tgaggaagtg tttttctgaa taatgtggtg tttatggcag ggtggagtat ttgttcaggg 360  
ccaggtagac tttgacccat tacgtggagg ttctgattac cgtgtttttt acctgaattt 420  
ccgcgtaccg tgtcaaagtc ttctgttttt acgtaggtgt cagctgatcg ctagggtatt 480  
tatacctcag ggtttgtgtc aagaggccac tcttgagtgc cagcgagaag agttttctcc 540

tctgcgcg	cagtttaata	ataaaaaaat	gagagatttg	cgatttctgc	ctcaggaaat	600
aatctctgct	gagactggaa	atgaaatatt	ggagcttg	gtgcacgccc	tgatgggaga	660
cgatccggag	ccacctgtgc	agctttttga	gcctcctacg	cttcaggaac	tgtatgattt	720
agaggtagag	ggatcggagg	attctaata	ggaagctgtg	aatggctttt	ttaccgattc	780
tatgctttta	gctgctaata	aaggattaga	attagatccg	cctttggaca	ctttcaatac	840
tccaggggtg	attgtggaaa	gcggtacagg	tgtaagaaaa	ttacctgatt	tgagttccgt	900
ggactgtgat	ttgactgtct	atgaagacgg	gtttcctccg	agtgtgagg	aggacctga	960
aaaggagcag	tccatgcaga	ctgcagcggg	tgaggagtg	aaggctgcc	atgttggtt	1020
tcagttggat	tgcccggagc	ttcctggaca	tggctgtaag	tcttgatga	ttcacaggaa	1080
aaatactgga	gtaaaggaac	tgttatgttc	gctttgttat	atgagaacgc	actgccactt	1140
tatttacagt	aagtgtgttt	aagttaaaat	ttaaagggaat	atgctgtttt	tcacatgtat	1200
attgagtgtg	agttttgtgc	ttcttattat	aggtcctgtg	tctgatgctg	atgaatcacc	1260
atctcctgat	tctactacct	cacctcctga	tattcaagca	cctgttcctg	tggaactgcg	1320
caagcccatt	cctgtgaagc	ttaagcctgg	gaaacgtcca	gcagtggaga	aacttgagga	1380
cttggttacag	ggtggggacg	gacctttgga	cttgagtaca	cggaaacgtc	caagacaata	1440
agtgttccat	atccgtgttt	acttaagggtg	acgtcaatat	ttgtgtgaga	gtgcaatgta	1500
ataaaaaat	gttaactgtt	cactggtttt	tattgctttt	tgggcgggga	ctcagggtata	1560
taagtagaag	cagacctgtg	tggttagctc	ataggagctg	gctttcatcc	atggagggtt	1620
gggccatttt	ggaagacctt	aggaagacta	ggcaactgtt	agagagcgct	tcggacggag	1680
tctccgggtt	ttggagattc	tggttcgcta	gtgaattagc	tagggtagtt	tttaggataa	1740
aacaggacta	taaacaagaa	tttgaaaagt	tggttgtaga	ttgcccagga	ctttttgaag	1800
ctcttaattt	gggccatcag	gttcacttta	aagaaaaagt	tttatcagtt	ttagactttt	1860
caaccccgag	tagaactgct	gctgctgtgg	cttttcttac	ttttatatta	gataaatgga	1920
tcccgcagac	tcatttcagc	aggggatacg	ttttggattt	catagccaca	gcattgtgga	1980
gaacatggaa	ggttcgcaag	atgaggacaa	tcttaggtta	ctggccagtg	cagccttttg	2040
gtgtagcggg	aatcctgagg	catccaccgg	tcactgccagc	ggttctggag	gaggaacagc	2100
aagaggacaa	cccagagacc	ggcctggacc	ctccagtggg	ggaggcggag	tagtgcactt	2160
gtctcctgaa	ctgcaacggg	tgcttactgg	atctacgtcc	actggacggg	ataggggctg	2220
taagagggag	agggcatcca	gtggtactga	tgctagatct	gagttggctt	taagtttaata	2280
gagtcgcaga	cgtcctgaaa	ccatttggtg	gcatgagggt	cagaaagagg	gaagggatga	2340
agtttctgta	ttgcaggaga	aatattcact	ggaacagggtg	aaaacatgtt	ggttggagcc	2400
agaggatgat	tgggcggtgg	ccattaaaaa	ttatgccaa	atagctttga	ggcctgataa	2460
acagtataag	atcagtagac	ggattaatat	ccggaatgct	tgttacatat	ctggaaatgg	2520
ggctgagggtg	gtaatagata	ctcaagacaa	gacagttatt	agatgctgca	tgatggatat	2580
gtggcctgga	gtagtcggta	tggaagcagt	cactttttgta	aatgttaagt	ttaggggaga	2640
tggttataat	ggaatagtgt	ttatggccaa	taccaaactt	atattgcatg	gttgtagctt	2700
ttttggtttc	aacaatacct	gtgtagatgc	ctggggacag	gttagtgtac	gggggtgtag	2760
tttctatgcg	tggttgattg	ccacagctgg	cagaaccaag	agtcaattgt	ctctgaagaa	2820
atgcatattc	caaagatgta	acctgggcat	tctgaatgaa	ggcgaagcaa	gggtccgtca	2880
ctgcgcttct	acagatactg	gatgttttat	tttaattaa	ggaaatgcca	gcgtaaagca	2940
taacatgatt	tgtggtgctt	ccgatgagag	gccttatcaa	atgctcactt	gtgctggtgg	3000
gcattgtaat	atgctggcta	ctgtgcatat	tgtttcccat	caacgcaaaa	aatggcctgt	3060
ttttgatcac	aatgtgttga	ccaagtgcac	catgcatgca	ggtgggcgta	gaggaatgtt	3120
tatgccttac	cagtgtaa	tgaatcatgt	gaaagtgttg	ttggaaccag	atgccttttc	3180
cagaatgagc	ctaacaggaa	tctttgacat	gaacacgcaa	atctggaaga	tcctgaggta	3240
tgatgatacg	agatcgaggg	tgcgcgcatg	cgaatgcgga	ggcaagcatg	ccagggtcca	3300
gccggtgtgt	gtagatgtga	ccgaagatct	cagaccggat	catttggtta	ttgcccgcac	3360
tgagcagag	ttcggatcca	gtggagaaga	aactgactaa	ggtgagtatt	gggaaaactt	3420
tggggtggga	ttttcagatg	gacagattga	gtaaaaattt	gttttttctg	tcttgacgt	3480
gacatgagt	gaaatgcttc	ttttaagggg	ggagtcttca	gcccttatct	gacagggcgt	3540
ctcccatcct	gggcaggagt	tcgtcagaat	gttatgggat	ctactgtgga	tggaagaccc	3600
gttcaacccg	ccaattcttc	aacgctgacc	tatgctaact	taagtcttct	acctttggac	3660
gcagctgcag	ccgctgccgc	cgctctgttc	gccgctaaca	ctgtgcttgg	aatgggttac	3720
tatggaagca	tcgtggctaa	ttccacttcc	tctaataacc	cttctacact	gactcaggac	3780
aagttacttg	tccttttggc	ccagctggag	gctttgaccc	aacgtctggg	tgaactttct	3840
cagcaggtgg	ccgagttgcg	agtacaaact	gagtcgtctg	tcggcacggc	aaagtctaaa	3900
taaaaaaaat	tccagaatca	atgaataaat	aaacgagctt	gttggtgatt	taaaatcaag	3960

tggtttttatt	tcattttttcg	cgcacgggtat	gccctggacc	accgatctcg	atcattgaga	4020
actcgggtgga	ttttttccag	aatcctatag	aggtgggatt	gaatgtttag	atacatgggc	4080
attaggccgt	ctttgggggtg	gagatagctc	cattgaagg	attcatgctc	cggggtagtg	4140
ttgtaaataca	cccagtcata	acaaggctcg	agtgcattgt	gttgacacat	atcttttaga	4200
agtaggctga	ttgccacaga	taagcccttg	gtgtagggtg	ttacaaaccg	gttgagctgg	4260
gaggggtgca	ttcgagggtga	aattatgtgc	attttggtt	ggatttttaa	gttggcaata	4320
ttgccgcaa	gatcccgctc	tgggttcattg	ttatgaagga	ctaccaagac	ggtgtatccg	4380
gtacatttag	gaaattttatc	gtgcagcttg	gatggaaaag	cgtggaaaaa	tttgagaca	4440
cccttggtgc	ctccgagatt	ttccatgcac	tcattccatga	taatagcaat	ggggccgtgg	4500
gcagcggcgc	gggcaaacac	gttccgtggg	tctgacacat	catagttagt	ttcctgagtt	4560
aaatcatcat	aagccatttt	aatgaatttg	gggcggagcg	taccagattg	gggtatgaat	4620
gttcccttcg	gccccggagc	atagtcccc	tcacagattt	gcatttccca	agctttcagt	4680
tctgaggggtg	ggaatcatgtc	cacctggggg	gctatgaaga	acaccgtttc	gggggccccg	4740
gtgattagtt	gggatgatag	caagtttctg	agcaattgag	atgtgccaca	tccgggtggg	4800
ccataaataa	ttccgattac	aggttgcagg	tggtagttaa	gggaacggca	actgccgtct	4860
tctcgaagca	agggggccac	ctcgttcattc	atttccctta	catgcattat	ttcccgacc	4920
aaatccatta	ggaggcgtc	tcctcctagt	gatagaagtt	cttgtagtga	ggaaaagt	4980
ttcagcgggt	ttagaccgtc	agccatgggc	attttgaaa	gagtttgctg	caaaagtct	5040
agtctgttcc	acagttcagt	gatgtgttct	atggcatctc	gatccagcag	acctcctcgt	5100
ttcgcgggtt	tggacggctc	ctggagtagg	gtatgagacg	atgggcgtcc	agcgtgcca	5160
gggttcgggtc	cttcagggt	ctcagtgttc	gagtcagggt	tggttccgtc	acagtgaagg	5220
ggtgtgcgcc	tgtttggg	cttgccagg	tgcgttcag	actcattctg	ctggtggaga	5280
acttctgtcg	cttgccgccc	tgtatgtcgg	ccaagtagca	gtttaccatg	agttcgtagt	5340
tgagcgccctc	ggctgcgtgg	cctttggcgc	ggagcttacc	tttggaagtt	ttcttgcata	5400
ccgggcagta	taggcatttc	agcgcataca	gcttgggcgc	aaggaaaatg	gattctgggg	5460
agtatgcatt	cgcgcgcag	gaggcgcaaa	cagtttcaca	ttccaccagc	caggttaaat	5520
ccggttcatt	ggggtcaaaa	acaagttttc	cgccattatt	tttgatgcgt	ttcttacctt	5580
tggctctccat	aagttcgtgt	cctcgttag	tgacaaacag	gctgtccgta	tctccgtaga	5640
ctgattttac	aggcctcttc	tccagtggag	tgcctcggtc	ttcttcgtac	aggaactctg	5700
accactctga	tacaaaggcg	cgcgtccagg	ccagcaca	ggaggctatg	tgggaggggt	5760
agcgatcggt	gtcaaccagg	gggtccacct	tttccaaagt	atgcaaacac	atgtcaccct	5820
cttcaacatc	caggaatgtg	attggcttgt	aggtgtattt	cacgtgacct	ggggtccccg	5880
ctgggggggt	ataaaagg	gcggttcttt	gctcttcctc	actgtcttcc	ggatcgctgt	5940
ccagggaacgt	cagctgttgg	ggtaggtatt	ccctctcgaa	ggcgggcatg	acctctgcac	6000
tcaggttgtc	agtttctaag	aacgaggagg	atttgatatt	gacagtgccg	gttgagatgc	6060
ctttcatgag	gttttcgtcc	atttggtcag	aaaacaca	ttttttattg	tcaagtttgg	6120
tggcaaatga	tccatacagg	gcgttgata	aaagtgttgc	aatggatcgc	atggtttgg	6180
tcttttccct	gtccgcgcgc	tctttggcgg	cgatgttgag	ttggacatac	tcgcgtgcca	6240
ggcacttcca	ttcggggaag	atagttgtta	attcatctgg	cacgattctc	acttgcacc	6300
ctcgattatg	caaggttaatt	aaatccacag	tgttggccac	ctcgcctcga	aggggttacc	6360
tgggtccaaca	gagcctacct	cctttcctag	aacagaaagg	gggaagtggg	tctagcataa	6420
gttcatcggg	agggctctga	tccatggtaa	agattcccgg	aagtaaatcc	ttatcaaaat	6480
agctgatggg	agtgggtgca	tctaaggcca	tttgccattc	tcgagctgcc	agtgcgcgct	6540
catatgggtt	aaggggactg	ccccagggca	tgggatgggt	gagagcagag	gcatacatgc	6600
cacagatgtc	atagacgtag	atgggatcct	caaagatgcc	tatgtagggt	ggatagcatc	6660
gccccctct	gatacttgct	cgcacatagt	catatagttc	atgtgatggc	gctagcagcc	6720
ccggacccaa	gttggtgcga	ttgggttttt	ctgttctgta	gacgatctgg	cgaaagatgg	6780
cgtgagaatt	ggaagagatg	gtgggtcttt	gaaaaatgtt	gaaatgggca	tgaggtagac	6840
ctacagagtc	tctgacaaag	tgggcataag	attcttgaag	cttggttacc	agttcggcgg	6900
tgacaagtac	gtctaggggc	cagtagtcaa	gtgtttcttg	aatgatgtca	taacctggtt	6960
gggttttctt	ttcccacagt	tcgcggttga	gaaggtattc	ttcgcgatcc	ttccagtact	7020
cttctagcgg	aaacccgtct	ttgtctgcac	ggtaagatcc	tagcatgtag	aactgattaa	7080
ctgccttgta	agggcagcag	cccttctcta	cgggtagaga	gtatgcttga	gcagcttttc	7140
gtagcggaagc	gtgagtaagg	gcaaagggtg	ctctgaccat	gactttgaga	aattgggtatt	7200
tgaagtccat	gtcgtcacag	gctccctgtt	cccagagttg	gaagtctacc	cgtttcttgt	7260
aggcgggggt	gggcaagcg	aaagtaacat	cattgaagag	aatcttaccg	gctctgggca	7320
taaaattg	agtgatgcgg	aaaggctgtg	gtacttccgc	tcgattgttg	atcacctggg	7380

cagctaggac	gatttcgctg	aaaccggtta	tgttggtgtcc	tacgatgtat	aattctatga	7440
aacgcggcgt	gcctctgacg	tgaggtagct	tactgagctc	atcaaagggt	aggtctgtgg	7500
ggtcagataa	ggcgtagtgt	tcgagagccc	attcgtgcag	gtgaggattt	gcatgtagga	7560
atgatgacca	aagatctacc	gccagtgctg	tttgtaactg	gtcccgatac	tgacgaaaat	7620
gccggccaat	tgccattttt	tctggagtga	cacagtagaa	ggttctgggg	tcttggtgcc	7680
atcgatccca	cttgagttta	atggctagat	cgtgggccat	gttgacgaga	cgctcttctc	7740
ctgagagttt	catgaccagc	atgaaaggaa	ctagttgttt	gccaaaggat	cccattccagg	7800
tgtaaagttc	cacatcgtag	gtcaggaaga	gtctttctgt	gcgaggatga	gagccgatcg	7860
ggaagaactg	gatttcctgc	caccagttgg	aggattggct	gttgatgtga	tggaagtaga	7920
agtttctgcg	gcgcgccgag	cattcgtggt	tgtgcttgta	cagacggccg	cagtagtcgc	7980
agcgttgcac	gggttgatc	tcgtgaatga	gctgtacctg	gcttcccttg	acgagaaatt	8040
tcagtgggaa	gccgaggcct	ggcgattgta	tctcgtgctc	ttctatatct	gctgtatcgg	8100
cctgttcac	ttctgtttcg	atggtggcta	tgctgacgag	cccccgcg	aggcaagttc	8160
agacctcggc	gcgggagggg	cggagctgaa	ggacgagagc	gcgcaggctg	gagctgtcca	8220
gagtcctgag	acgctgcgga	ctcaggttag	taggtaggga	cagaagatta	acttgcatga	8280
tcttttccag	ggcgtgcggg	aggttcagat	ggtacttgat	ttccacaggt	tcgtttgtag	8340
agacgtcaat	ggcttgccag	gttcctgtgc	ctttgggcgc	cactaccgta	cctttgtttt	8400
ttcttttgat	cggtggtggc	tctcttgctt	cttgcatgct	cagaagcggg	gacggggacg	8460
cgcgccgggc	ggcagcgggt	gttcgggacc	cgggggcatg	gctggttagt	gcacgtcggc	8520
gccgcgcacg	ggcaggttct	ggtattgcgc	tctgagaaga	cttgctgctg	ccaccacgcg	8580
tcgattgacg	tcttgatctt	gacgtctctg	ggtgaaagct	accggccccg	tgagcttgaa	8640
cctgaaagag	agttcaacag	aatcaatttc	ggtatcgtta	acggcagctt	gtctcagtat	8700
ttcttgtagc	tcaccagagt	tgtcctggta	ggcgatctcc	gccatgaact	gctcgatttc	8760
ttcctcctga	agatctccgc	gacccgctct	ttcgacgggtg	gccgcgaggt	cattggagat	8820
acggcccatg	agttgggaga	atgcattcat	gcccgcctcg	ttccagacgc	ggctgtaaac	8880
cacggccccc	tcggagtctc	ttgcgcgcac	caccacctga	gcgagggtta	gctccacgtg	8940
tctggtgaag	accgcatagt	tgcataggcg	ctgaaaaagg	tagttgagtg	tggtggcaat	9000
gtgttcggcg	acgaagaaat	acatgatcca	tcgtctcagc	ggcatttctg	taacatcgcc	9060
cagagcttcc	aagcgctcca	tggcctcgta	gaagtccacg	gcaaaaattaa	aaaactggga	9120
ggttcgcgcg	gacacgggtca	attcctcctc	gagaagacgg	atgagttcgg	ctatggtggc	9180
ccgtacttcg	cggtcgaagg	ctcccgggat	ctcttcttcc	tcttctatct	cttcttccac	9240
taacatctct	tcttcgtctt	caggcggggg	cgaggggggc	acgcggcgac	gtcgacggcg	9300
cacgggcaaa	cggtcgatga	atcgttcaat	gacctctccg	cggcggcggc	gcatggtttc	9360
agtgaaggcg	cgcccggttct	cgcgcggtcg	cagagtaaaa	acaccgccgc	gcatctcctt	9420
aaagtgggtga	ctgggaggtt	ctccgtttgg	gaggagagag	gcgctgatta	tacattttat	9480
taattggccc	gtagggactg	cgcgagagag	tctgatcggt	tcaagatcca	cgggatctga	9540
aaacctttcg	acgaaagcgt	ctaaccagtc	acagtcacaa	ggtaggctga	gtacggcttc	9600
ttgtggggcg	gggtgggttat	gtgttcggtc	tgggtcttct	gtttcttctt	catctcgggg	9660
aggtgagacg	atgctgctgg	tgatgaaatt	aaagtaggca	gttctaagac	ggcggtggtt	9720
ggcgaggagc	accaggtctt	tgggtccggc	ttgctggata	cgaggcgat	tgccatttcc	9780
ccaagcatta	tcttgacatc	tagcaagatc	tttgtagtag	tcttgcatga	gccgttctac	9840
gggcacttct	tcttcacccg	ttctgccatg	catacgtgtg	agtccaaatc	cgcgcatgtg	9900
ttgtaccagt	gccaagtcag	ctacgactct	ttcggcgagg	atggcttgct	gtacttgggt	9960
aagggtggct	tgaagtcac	caaaatccac	aaagcggtgg	taagcccctg	tattaatggt	10020
gtaagcacag	ttggccatga	ctgaccagtt	aactgtctgg	tgaccagggc	gcacgagctc	10080
ggtgtattta	aggcggaat	aggcgcggtt	gtcaaagatg	taatcgttgc	aggtgcgcac	10140
cagatactgg	taccctataa	gaaaatgcgg	cggtgggttg	cggtagagag	gccatcgttc	10200
tgtagctgga	gcgccagggg	cgaggtcttc	caacataagg	cggtgatagc	cgtagatgta	10260
cctggacatc	caggtgattc	ctgcggcggt	agtagaagcc	cgaggaaact	cgcgtagcgc	10320
gttccaaatg	ttgcgtagcg	gcatgaagta	gttcattgta	ggcacgggtt	gaccagttag	10380
gcgcgcgcag	tcattgatgc	tctatagaca	cggagaaaaa	gaaagcggtc	agcgactcga	10440
ctccgtagcc	tgagggaacg	tgaacgggtt	gggtcgcggt	gtaccccggt	tcgagacttg	10500
tactcgagcc	ggccggagcc	gcggctaacg	tggtattggc	actcccgtct	cgaccagacc	10560
tacaaaaatc	caggatacgg	aatcgagtcg	ttttgctggt	ttccgaatgg	caggggaagt	10620
agtcctatct	tttttttttt	tttgccgctc	agatgcatcc	cgtgctgcga	cagatgcgcc	10680
cccaacaaca	gccccctcg	cagcagcagc	agcagcaacc	acaaaaggct	gtccctgcaa	10740
ctactgcaac	tgccgccgtg	agcgggtgcg	gacagcccgc	ctatgatctg	gacttggaag	10800

agggcgaagg	actggcacgt	ctaggtgcgc	cttcgcccga	gcggcatccg	cgagttcaac	10860
tgaaaaaaga	ttctcgcgag	gcgtatgtgc	cccaacagaa	cctatttaga	gacagaagcg	10920
gcgaggagcc	ggaggagatg	cgagcttccc	gctttaacgc	gggtcgtgag	ctgcgtcacg	10980
gtttggaccg	aagacgagtg	ttgcgagacg	aggatttcga	agttgatgaa	gtgacaggga	11040
tcagtcctgc	cagggcacac	gtggctgcag	ccaaccttgt	atcggcttac	gagcagacag	11100
taaaggaaga	gcgtaacttc	caaaagtctt	ttaataatca	tgtgcgaacc	ctgattgccc	11160
gcgaagaagt	tacccttggt	ttgatgcatt	tgtgggattt	gatggaagct	atcattcaga	11220
accctactag	caaacctctg	accgcccagc	tgtttctggt	ggtgcaacac	agcagagaca	11280
atgaggcttt	cagagaggcg	ctgctgaaca	tcaccgaacc	cgaggggaga	tggttgtatg	11340
atcttatcaa	cattctacag	agtatcatag	tgcaggagcg	gagcctgggc	ctggccgaga	11400
aggtagctgc	catcaattac	tcggttttga	gcttgggaaa	atattacgct	cgcaaaatct	11460
acaagactcc	atacgttccc	atagacaagg	aggtgaagat	agatgggttc	tacatgcgca	11520
tgacgctcaa	ggtcttgacc	ctgagcgatg	atcttggggg	gtatcgcaat	gacagaatgc	11580
atcgcgcggt	tagcgccagc	aggaggcgcg	agttaagcga	cagggaaactg	atgcacagtt	11640
tgcaaagagc	tctgactgga	gctggaaccg	agggtgagaa	ttacttcgac	atgggagctg	11700
acttgacgtg	gcagcctagt	cgcagggctc	tgagcgccgc	gacggcagga	tgtgagcttc	11760
cttacataga	agaggcggt	gaaggcgagg	aggaagaggg	cgagtacttg	gaagactgat	11820
ggcacaaccc	gtgttttttg	ctagatggaa	cagcaagcac	cggatcccgc	aatgcgggcg	11880
gcgctgcaga	gccagccgtc	cggcattaac	tcctcggacg	attggacca	ggccatgcaa	11940
cgtatcatgg	cgttgacgac	tcgcaacccc	gaagccttta	gacagcaacc	ccaggccaac	12000
cgtctatcgg	ccatcatgga	agctgtagt	ccttcccgat	ctaattcccac	tcatgagaag	12060
gtcctggcca	tcgtgaacgc	gttgggtggag	aacaaagcta	ttcgtccaga	tgaggccgga	12120
ctggtataca	acgctctctt	agaacgcgtg	gctcgctaca	acagtagcaa	tgtgcaaacc	12180
aatttgacc	gtatgataac	agatgtacgc	gaagccgtgt	ctcagcgcg	aaggttccag	12240
cgtgatgcca	acctgggttc	gctgggtggc	ttaaagtctt	tcttgagtac	tcagcctgct	12300
aatgtgccgc	gtgggtcaaca	ggattatact	aactttttta	gtgctttgag	actgatggta	12360
tcagaagtc	ctcagagcga	agtgtatcag	tccggtcctg	attacttctt	tcagactagc	12420
agacagggtc	tgcagacggt	aaatctgagc	caagctttta	aaaaccttaa	aggtttgtgg	12480
ggagtgcatt	ccccggtagg	agaaagagca	accgtgtcta	gcttggttaac	tccgaactcc	12540
cgctgttat	tactgttggt	agctcctttc	accgacagcg	gtagcatcga	ccgtaattcc	12600
tatttggtt	acctactaaa	cctgtatcgc	gaagccatag	ggcaaagtca	ggtggacgag	12660
cagacctatc	aagaaattac	ccaagtcagt	cgcgcttttg	gacaggaaga	cactggcagt	12720
ttggaagcca	ctctgaactt	cttgcttacc	aatcggtctc	aaaagatccc	tctcaatat	12780
gctcttactg	cggaggagga	gaggatcctt	agatatgtgc	agcagagcgt	gggattgttt	12840
ctgatgcaag	agggggcaac	tccgactgca	gcactggaca	tgacagcgcg	aaatatggag	12900
cccagcatgt	atgccagtaa	ccgacctttc	attaacaaac	tgctggacta	cttgccacaga	12960
gctgccgcta	tgaactctga	ttatttcacc	aatgccatct	taaacccgca	ctggctgccc	13020
ccacctggtt	tctacacggg	cgaatatgac	atgcccagac	ctaatagacg	atctctgtgg	13080
gacgacgtgg	acagcgatgt	tttttcacct	ctttctgac	atcgacgtg	gaaaaaggaa	13140
ggcgggtgata	gaatgcattc	ttctgcactg	ctgtccgggg	tcattgggtg	taccgcggtc	13200
gagccccagt	ctgcaagtcc	ttttcctagt	ctacctttt	ctctacacag	tgtactgtag	13260
agcgaagtgg	gtagaataag	tcgcccaggt	ttaatgggcg	aagaggagta	cctaacacgt	13320
tccttgctca	gaccggcaag	agaaaaaaat	ttcccaaaca	atggaataga	aagtttggtg	13380
gataaaatga	gtagatggaa	gacttatgct	caggatcaca	gagacgagcc	tgggatcatg	13440
gggactacaa	gtagagcgag	ccgtagacgc	cagcgccatg	acagacagag	gggtcttgtg	13500
tgggacgatg	aggattcggc	cgatgatagc	agcgtgttgg	acttgggtgg	gagagggaagg	13560
ggcaaccctg	ttgctcattt	gcgccctcgc	ttgggtggta	tgttgtgaaa	aaaaataaaa	13620
aagaaaaact	caccaaggcc	atggcgacga	gcgtacgttc	gttcttcttt	attatctgtg	13680
tctagtataa	tgaggcgagt	cgtgctaggc	ggagcggtgg	tgtatccgga	gggtcctcct	13740
ccttcgtacg	agagcgtgat	gcagcagcag	caggcgacgg	cggtgatgca	atccccactg	13800
gaggctccct	ttgtgcctcc	gcgatacctg	gcacctacgg	agggcagaaa	cagcattcgt	13860
tactcggaac	tggcacctca	gtacgatacc	accaggttgt	atctgggtgga	caacaagtcg	13920
gcggacattg	cttctctgaa	ctatcagaat	gaccacagca	acttcttgac	cacggtgggtg	13980
cagaacaatg	actttacccc	tacggaagcc	agcaccacga	ccattaactt	tgatgaacga	14040
tcgcggtggg	gcggtcagct	aaagaccatc	atgcatacta	acatgccaaa	cgtgaacgag	14100
tatatgttta	gtaacaagtt	caaagcgcgt	gtgatggtgt	ccagaaaacc	tcccagcggt	14160
gctgcagttg	gggatactta	tgatcacaag	caggatattt	tggaatatga	gtggttcgag	14220

tttactttgc	cagaaggcaa	cttttcagtt	actatgacta	ttgatttgat	gaacaatgcc	14280
atcatagata	attacttgaa	agtgggtaga	cagaatggag	tgcttgaaag	tgacattggt	14340
gttaagttcg	acaccaggaa	cttcaagctg	ggatgggata	ccgaaaccaa	gttgatcatg	14400
cctggagtgt	atacgtatga	agccttccat	cctgacattg	tcttactgcc	tggctgcgga	14460
gtggatttta	ccgagagtcg	tttgagcaac	cttcttggtg	tcagaaaaaa	acagccattt	14520
caagagggtt	ttaagatttt	gtatgaagat	ttagaagggtg	gtaatatattcc	ggccctcttg	14580
gatgtagatg	cctatgagaa	cagtaagaaa	gaacaaaaag	ccaaaataga	agctgctaca	14640
gctgctgcag	aagctaaggc	aaacatagtt	gccagcgact	ctacaagggt	tgctaacgct	14700
ggagagggtca	gaggagacaa	ttttgcgcca	acacctgttc	cgactgcaga	atcattattg	14760
gccgatgtgt	ctgaagggaac	ggacgtgaaa	ctcactattc	aacctgtaga	aaaagatagt	14820
aagaatagaa	gctataatgt	gttggaagac	aaaatcaaca	cagcctatcg	cagttggtat	14880
ctttcgtaca	attatggcga	tcccgaaaaa	ggagtgcggt	cctggacatt	gctcaccacc	14940
tcagatgtca	cctgcgggagc	agagcaggtt	tactggtcgc	ttccagacat	gatgaaggat	15000
cctgtcactt	tccgctccac	tagacaagtc	agtaactacc	ctgtggtggg	tgagagcgtt	15060
atgcccgtct	tctcaaagag	cttctacaac	gaacaagctg	tgtactccca	gcagctccgc	15120
cagtccacct	cgcttacgca	cgcttccaac	cgctttcctg	agaaccagat	tttaatccgt	15180
ccgccggcgc	ccaccattac	caccgtcagt	gaaaacgttc	ctgctctcac	agatcacggg	15240
accctgccgt	tgcgcagcag	tatccgggga	gtccaacgtg	tgaccgttac	tgacgccaga	15300
cgccgcacct	gtccctacgt	gtacaaggca	ctgggcatag	tcgcaccgcg	cgctcctttca	15360
agccgcactt	tctaaaaaaa	aaaaatgtcc	attcttatct	cgcccagtaa	taacaccggt	15420
tgggggtctg	gcgctccaag	caagatgtac	ggaggcgcac	gcaaacgttc	tacccaacat	15480
cccgtgcgtg	ttcgcggaca	ttttcgcgct	ccatgggggtg	ccctcaaggg	ccgcaactcg	15540
gttcgaacca	ccgtcgatga	tgtaatcgat	caggtggttg	ccgacgcccg	taattatact	15600
cctactgcgc	ctacatctac	tgtggatgca	gttattgaca	gtgtagtggc	tgacgctcgc	15660
aactatgctc	gacgtaagag	ccggcgaaag	cgcatcgcca	gacgccaccg	agctaccact	15720
gccatgcgag	ccgcaagagc	tctgctacga	agagctagac	gcgtggggcg	aagagccatg	15780
cttagggcgg	ccagacgtgc	agcttcgggc	gccagcgccg	gcaggtcccg	caggcaagca	15840
gccgctgtcg	cagcggcgac	tattgccgac	atggcccaat	cgcgaaagag	caatgtatac	15900
tgggtgcgtg	acgctgccac	cgggtcaacgt	gtaccctgtc	gcaccctgcc	ccctcgcact	15960
tagaagatac	tgagcagttc	ccgatgttgt	gtcccagcgg	cgaggatgtc	caagcgcaaa	16020
tacaaggaag	aaatgctgca	ggttatcgca	cctgaagtct	acggccaacc	gttgaaggat	16080
gaaaaaaaac	cccgcaaaat	caagcggggt	aaaaaggaca	aaaaagaaga	ggaagatggc	16140
gatgatgggc	tggcggagtt	tgtgcgcgag	tttgccccac	ggcgacgcgt	gcaatggcgt	16200
gggcgcaaa	ttcgacatgt	gttgagacct	ggaacttcgg	tggctctttac	acccggcgag	16260
cgttcaagcg	ctacttttaa	gcgttcctat	gatgaggtgt	acggggatga	tgatattctt	16320
gagcaggcgg	ctgaccgatt	aggcgagttt	gcttatggca	agcgtagtag	aataacttcc	16380
aaggatgaga	cagtgtcaat	acccttggat	catggaaatc	ccacccttag	tcttaaaccg	16440
gtcactttgc	agcaagtgtt	acccgtaact	ccgcgaacag	gtgttaaacy	cgaaggtgaa	16500
gatttgtatc	ccactatgca	actgatggta	cccaaaccgc	agaagttgga	ggcagtttg	16560
gagaaagtaa	aagtggatcc	agatattcaa	cctgaggtta	aagtgagacc	cattaagcag	16620
gtagcgctcg	gtctgggggt	acaaactgta	gacattaaga	ttcccactga	aagtatggaa	16680
gtgcaaaactg	aaccgcgcaa	gcctactgcc	acctccactg	aagtgcacac	ggatccatgg	16740
atgcccattgc	ctattacaac	tgacgccgcc	ggtcccactc	gaagatcccg	acgaaagtac	16800
ggtccagcaa	gtctgttgat	gcccaattat	gttgtaacac	catctattat	tcctactcct	16860
ggttaccgag	gcactcgcta	ctatcgagc	cgaaacagta	cctcccgcgc	tcgccgcaag	16920
acacctgcaa	atcgagtcg	tcgccgtaga	cgcacaagca	aaccgactcc	cggcgccctg	16980
gtgcggcaag	tgtaccgcaa	tggtagtgcg	gaacctttga	cactgccgcg	tgcgcggttac	17040
catccgagta	tcatacactta	atcaatgttg	ccgctgcctc	cttgacagata	tggccctcac	17100
ttgtcgcctt	cgcgttccca	tactgggtta	ccgaggaaga	aactcgcgcc	gtagaagagg	17160
gatgttggga	cgcggaatgc	gacgtacag	gcgacggcgt	gctatccgca	agcaattgcg	17220
gggtggtttt	ttaccagcct	taattccaat	tatcgctgct	gcaattggcg	cgataccagg	17280
catagcttcc	gtggcggttc	aggcctcgca	acgacattga	cattggaaaa	aaaacgtata	17340
aataaaaaaa	aatacaatgg	actctgacac	tcctggtcct	gtgactatgt	tttcttagag	17400
atggaagaca	tcaatttttc	atccttggtc	ccgcgacacg	gcacgaagcc	gtacatgggc	17460
acctggagcg	acatcggcac	gagccaactg	aacggggggcg	ccttcaattg	gagcagtatc	17520
tggagcgggc	ttaaaaaattt	tggctcaacc	ataaaaacat	acgggaacaa	agcttggaaac	17580
agcagtagag	gacaggcgct	tagaaataaa	cttaaagacc	agaacttcca	acaaaaagta	17640



gtcgatggga tagcttccgg catcaatgga gtggtagatt tggctaacca ggctgtgcag 17700  
 aaaaagataa acagtcgttt ggacccgccg ccagcaaccc caggtgaaat gcaagtggag 17760  
 gaagaaattc ctccgccaga aaaacgaggc gacaagcgtc cgcgtcccga tttggaagag 17820  
 acgctggtga cgcgcgtaga tgaaccgcct tcttatgagg aagcaacgaa gcttggaaatg 17880  
 cccaccacta gaccgatagc cccaatggcc accgggggtga tgaaaccttc tcagttgcat 17940  
 cgacccgtca ccttggattt gccccctccc cctgctgcta ctgctgtacc cgcttctaag 18000  
 cctgtcgtctg ccccgaaacc agtcgccgta gccaggtcac gtcccggggg cgctcctcgt 18060  
 ccaaattgctc actggcaaaa tactctgaac agcatcgtgg gtctaggcgt gcaaagtgtg 18120  
 aaacgccgtc gctgctttta attaaatatg gtagtagcgt taacttgcct atctgtgtat 18180  
 atgtgtcatt acacgccgtc acagcagcag aggaaaaaag gaagaggtcg tgcgtcgacg 18240  
 ctgagttact ttcaagatgg ccaccccatc gatgctgccc caatgggcat acatgcacat 18300  
 cgccggacag gatgcttcgg agtacctgag tccgggtctg gtgcagttcg cccgcgccac 18360  
 agacacctac ttcaatctgg gaaataagtt tagaaatccc accgtagcgc cgaccacga 18420  
 tgtgaccacc gaccgtagcc agcggctcat gttgcgcttc gtgcccggtg accgggagga 18480  
 caatacatac tcttaciaaag tgcggtacac cctggccgtg ggcgacaaca gagtgtgga 18540  
 tatggccagc acgttctttg acattagggg cgtgttgagc agaggtccca gtttcaaacc 18600  
 ctattctggt acggcttaca actctctggc tcttaaaggc gctccaaatg catctcaatg 18660  
 gattgcaaaa ggctgtacaa ctgcagcagc cgcaggcaat ggtgaagaag aacatgaaac 18720  
 agaggagaaa actgctactt acacttttgc caatgctcct gtaaaagccg aggtcctaat 18780  
 tacaaaagag ggcttaccaa taggtttgga gatttcagct gaaaacgaat ctaaacccat 18840  
 ctatgcagat aaactttatc agccagaacc tcaagtggga gatgaaactt ggactgacct 18900  
 agacggaaaa accgaagagt atggaggcag ggctctaaag cctactacta acatgaaacc 18960  
 ctgttacggg tcttatgcga agcctactaa tttaaaagggt ggtcaggcaa aaccgaaaaa 19020  
 ctccgaaccg tcgagtgaag aaattgaata tgatattgac atggaatttt ttgataactc 19080  
 atcgcaaaag acaaaacttca gtcctaaaaa tgtcatgtat gcagaaaatg taggtttgga 19140  
 aacgccagac actcatgtag tgtacaaacc tggaacagaa gacacaagtt ccgaagctaa 19200  
 tttgggacaa cagtctatgc ccaacagacc caactacatt ggcttcagag ataactttat 19260  
 tggactcatg tactataaca gtactggtaa catgggggtg ctggctgggtc aagcgtctca 19320  
 gttaaattgca gtggttgact tgcaggacag aaacacagaa ctttcttacc aactcttgct 19380  
 tgactctctg ggcgacagaa ccagatactt tagcatgtgg aatcaggctg tggacagtta 19440  
 tgatcctgat gtacgtgtta ttgaaaatca tgggtgtgga gatgaacttc ccaactattg 19500  
 ttttccactg gacggcatag gtgttccaac aaccagttac aaatcaatag ttccaaatgg 19560  
 agaagataat aataattgga aagaacctga agtaaatgga acaagtgaga tcggacaggg 19620  
 taatttggtt gccatggaaa ttaaccttca agccaatcta tggcgaagtt tcttttattc 19680  
 caatgtggct ctgtatctcc cagactcgta caaatacacc ccgtccaatg tcaactcttc 19740  
 agaaaacaaa aacacctacg actacatgaa cggggcgggtg gtgccgccat ctctagtaga 19800  
 cacctatgtg aacattgggt ccaggtgggtc tctggatgcc atggacaatg tcaacccatt 19860  
 caaccaccac cgtaacgctg gcttgcgtta ccgatctatg cttctgggtg acggacgtta 19920  
 tgtgcctttc cacatacaag tgcctcaaaa attcttcgct gttaaaaacc tgctgcttct 19980  
 cccaggctcc tacacttatg agtggaactt taggaaggat gtgaacatgg ttctacagag 20040  
 ttccctcggt aacgacctgc gggtagatgg cgccagcatc agtttcacga gcatcaacct 20100  
 ctatgctact tttttcccca tggctcacia caccgcttcc acccttgaag ccactgtcgc 20160  
 gaatgacacc aatgatcagt cattcaacga ctacctatct gcagctaaca tgctctaccc 20220  
 cattcctgcc aatgcaacca atattcccat ttccattcct tctcgcaact gggcggtttt 20280  
 cagaggctgg tcattttacca gactgaaaac caaagaaact ccctcttttg ggtctggatt 20340  
 tgacccttac tttgtctatt ctggttctat tccctacctg gatggtacct tctacctgaa 20400  
 ccacactttt aagaaggttt ccatcatgtt tgactcttca gtgagctggc ctggaaatga 20460  
 caggttacta tctcctaacg aatttgaaat aaagcgcact gtggatggcg aaggctacaa 20520  
 cgtagcccaa tgcaacatga ccaaagactg gttcttggtg cagatgctcg ccaactacaa 20580  
 catcggctat cagggcttct acattccaga aggatacaaa gatcgcagtgt attcattttt 20640  
 cagaaacttc cagcccatga gcaggcaggt ggttgatgag gtcaattaca aagacttcaa 20700  
 ggccgtcgcc ataccctacc aacacaacaa ctctggcttt gtgggttaca tggctccgac 20760  
 catgcgccaa ggtcaaccct atcccgttaa ctatccctat ccactcattg gaacaactgc 20820  
 cgtaaatagt gttacgcaga aaaagttctt gtgtgacaga accatgtggc gcataccgtt 20880  
 ctcgagcaac ttcattgtct tgggggccct tacagacttg ggacagaata tgctctatgc 20940  
 caactcagct catgctctgg acatgacctt tgaggtggat cccatggatg agcccacct 21000  
 gctttatctt ctcttcgaag ttttcgacgt ggtcagagtg catcagccac accgcggcat 21060

catcgaggca	gtctacctgc	gtacaccgtt	ctcggccggt	aacgctacca	cgtaagaagc	21120
ttcttgcttc	ttgcaaatag	cagctgcaac	catggcctgc	ggatcccaaa	acggctccag	21180
cgagcaagag	ctcagagcca	ttgtccaaga	cctgggttgc	ggaccctatt	ttttgggaac	21240
ctacgataag	cgcttcccgg	ggttcatggc	ccccgataag	ctcgcctgtg	ccattgtaaa	21300
tacggccgga	cgtgagacgg	ggggagagca	ctggttggct	ttcggttgga	acccacgttc	21360
taacacctgc	tacctttttg	atccttttgg	attctcggat	gacgtctca	aacagattta	21420
ccagtttgaa	tatgagggtc	tcctgcgcgg	cagcgtctct	gctaccaagg	accgctgtat	21480
tacgttgga	aaatctaccc	agaccgtgca	gggcccccg	tctgccgcct	gcggactttt	21540
ctgctgcatg	ttccttcacg	cctttgtgca	ctggcctgac	cgtcccatgg	acggaaaccc	21600
caccatgaaa	ttgctaactg	gagtgccaaa	caacatgctt	cattctccta	aagtccagcc	21660
caccctgtgt	gacaatcaaa	aagcactcta	ccattttctt	aataccatt	cgcttatttt	21720
tgcgtctcat	cgtaacacaca	tcgaaagggc	cactgcgttc	gaccgtatgg	atgttcaata	21780
atgactcatg	taaacacacg	gttcaataaa	catcacttta	tttttttaca	tgtatcaagg	21840
ctctggatta	cttattttatt	tacaagtcga	atgggttctg	acgagaatca	gaatgacccg	21900
caggcagtga	tacgttgcg	aactgatact	tgggttgcca	cttgaattcg	ggaatcacca	21960
acttggaac	cggtatatcg	ggcaggatgt	cactccacag	ctttctggtc	agctgcaaag	22020
ctccaagcag	gtcaggagcc	gaaatcttga	aatcacaatt	aggaccagt	ctctgagcgc	22080
gagagttg	gtacaccgga	ttgcagcaact	gaaacaccat	cagcgacgga	tgtctcacgc	22140
ttgccagcac	ggtgggatct	gcaatcatgc	ccacatccag	atcttcagca	ttggcaatgc	22200
tgaacggggt	catcttgca	gtctgcctac	ccatggcggg	cacccaatta	ggcttgtggt	22260
tgcaatcgca	gtgcaggggg	atcagtatca	tcttggcctg	atcctgtctg	attcctggat	22320
acacggctct	catgaaagca	tcatattgct	tgaagcctg	ctgggcttta	ctaccctcgg	22380
tataaaacat	cccgaggagc	ctgctcgaaa	actggttagc	tgcacagccg	gcatcatcca	22440
cacagcagcg	ggcgtcattg	ttggctattt	gcaccacact	tctgccccag	cggttttggg	22500
tgattttggt	tcgctcgggg	ttctccttta	aggctcggtg	tccgttctcg	ctggccacat	22560
ccatctcgat	aatctgctcc	ttctgaatca	taatattgcc	atgcaggcac	ttcagcttgc	22620
cctcataatc	attgcagcca	tgaggccaca	acgcacagcc	tgtacattcc	caattatggt	22680
gggcgatctg	agaaaaagaa	tgtatcattc	cctgcagaaa	tcttcccatc	atcgtgctca	22740
gtgtcttgtg	actagtga	gttaactgga	tgcctcggtg	ctcttcgttt	acgtactggt	22800
gacagatg	cttgtattgt	tcgtgttgct	caggcattag	tttaaaacag	gttctaagtt	22860
cgttatccag	cctgtacttc	tccatcagca	gacacatcac	ttccatgcct	ttctcccaag	22920
cagacaccag	gggcaagcta	atcggattct	taacagtgca	ggcagcagct	ccttttagcca	22980
gagggtcac	tttagcgatc	ttctcaatgc	ttcttttgcc	atccttctca	acgatgcgca	23040
cgggcgggta	gctgaaaccc	actgctacaa	gttgcgccctc	ttctctttct	tcttcgctgt	23100
cttgactgat	gtcttgcatg	gggatatgtt	tggcttctct	tggcttcttt	ttggggggta	23160
tcggaggagg	aggactgtcg	ctccgttccg	gagacaggga	ggattgtgac	gtttcgctca	23220
ccattaccaa	ctgactgtcg	gtagaagaac	ctgacccac	acggcgacag	gtgtttttct	23280
tcggggggcag	aggtggaggc	gattgcgaag	ggctgcggtc	cgacctggaa	ggcggtgac	23340
tggcagaacc	ccttcgcgt	tcgggggtg	gctccctgtg	gcggctcgctt	aactgatttc	23400
cttcgcggct	ggccattgtg	ttctcctagg	cagagaaaca	acagacatgg	aaactcagcc	23460
attgctgtca	acatgccac	gagtgccatc	acatctcgtc	ctcagcgacg	aggaaaagga	23520
gcagagctta	agcattccac	cgccagtc	tgccaccacc	tctaccctag	aagataagga	23580
ggtcgacgca	tctcatgaca	tgcagaataa	aaaagcgaaa	gagtctgaga	cagacatcga	23640
gcaagaccg	ggctatgtga	caccggtgga	acacgaggaa	gagttgaaac	gctttctaga	23700
gagagaggat	gaaaactgcc	caaaacagcg	agcagataac	tatcaccaag	atgctggaaa	23760
tagggatcag	aacaccgact	acctcatagg	gcttgacggg	gaagacgcgc	tccttaaaca	23820
tctagcaaga	cagtcgctca	tagtcaagga	tgcattattg	gacagaactg	aagtgcccat	23880
cagtgtggaa	gagctcagct	gcgcctacga	gcttaacctt	ttttcacctc	gtactcccc	23940
caaacgtcag	caaacggca	cctgcgagcc	aaatcctcgc	ttaaactttt	atccagcttt	24000
tgctgtgcca	gaagtactgg	ctacctatca	catctttttt	aaaaatcaaa	aaattccagt	24060
ctcctgccgc	gctaategca	cccgcgcgca	tgcctactc	aatctgggac	ctggttcacg	24120
cttacctgat	atagcttctt	tgggaagagg	tccaaagatc	ttcgagggtc	tgggcaataa	24180
tgagactcgg	gccgcaaatg	ctctgcaaaa	gggagaaaat	ggcatggatg	agcatcacag	24240
cgttctggtg	gaattggaag	gcgataatgc	cagactcgca	gtactcaagc	gaagcgtcga	24300
ggtcacacac	ttcgcatatc	ccgctgtcaa	cctgccccct	aaagtcatga	cggcggtcat	24360
ggaccagtta	ctcattaagc	gcgcaagtcc	cctttcagaa	gacatgcatg	acccagatgc	24420
ctgtgatgag	ggtaaacccag	tggtcagtga	tgagcagcta	acccgatggc	tgggcaccga	24480

ctctccccg	gatttggaag	agcgtcgcaa	gcttatgatg	gccgtggtgc	tggttaccgt	24540
agaactagag	tgtctccgac	gtttctttac	cgattcagaa	accttgcgca	aactcgaaga	24600
gaatctgcac	tacactttta	gacacggctt	tgtgcggcag	gcatgcaaga	tatctaacgt	24660
ggaactcacc	aacctgggtt	cctacatggg	tattctgcat	gagaatcgcc	taggacaaag	24720
cgtgctgcac	agcaccctta	agggggaagc	ccgccgtgat	tacatccgcg	attgtgtcta	24780
tctctacctg	tgccacacgt	ggcaaaccgg	catgggtgta	tggcagcaat	gtttagaaga	24840
acagaacttg	aaagagcttg	acaagctctt	acagaaatct	cttaagggtt	tgtggacagg	24900
gttcgacgag	cgcaccgtcg	cttccgacct	ggcagacctc	atcttcccag	agcgtctcag	24960
ggttactttg	cgaaaacggat	tgcctgactt	tatgagccag	agcatgctta	acaattttcg	25020
ctctttcatc	ctggaacgct	ccggtatcct	gcccgccacc	tgctgcgcac	tgccctccga	25080
ctttgtgcct	ctcacctacc	gcgagtggcc	cccgccgcta	tggagtcaact	gctacctgtt	25140
ccgtctggcc	aactatctct	cctaccactc	ggatgtgatc	gaggatgtga	gcggagacgg	25200
cttgctggag	tgccactgcc	gctgcaatct	gtgcacgccc	caccgggtccc	tagcttgcaa	25260
ccccagttg	atgagcgaaa	cccagataat	aggcaccttt	gaattgcaag	gccccagcag	25320
ccaaggcgat	gggtcttctc	ctgggcaaa	tttaaaactg	accccgggac	tgtggacctc	25380
cgcctacttg	cgcaagtttg	ctccggaaga	ttaccacccc	tatgaaatca	agttctatga	25440
ggaccaatca	cagcctccaa	aggccgaact	ttcggttgc	gtcatcacc	agggggcaat	25500
tctggcccaa	ttgcaagcca	tccaaaaatc	ccgccaaaga	tttctactga	aaaagggtaa	25560
gggggtctac	cttgaccccc	agaccggcga	ggaactcaac	acaaggttcc	ctcaggatgt	25620
cccaacgacg	agaaaacaag	aagttgaagg	tgcagccgcc	gccccagaa	gatattggagg	25680
aagattggga	cagtcaggca	gaggaggcgg	aggaggacag	tctggaggac	agtctggagg	25740
aagacagttt	ggaggaggaa	aacgaggagg	cagaggagggt	ggaagaagta	accgccgaca	25800
aacagttatc	ctcggctgcg	gagacaagca	acagcgctac	catctccgct	ccgagtcgag	25860
gaaccggcg	gcgtcccagc	agtagatggg	acgagaccgg	acgcttcccg	aaccacaacca	25920
gcgcttccaa	gaccggtaag	aaggatcggc	agggatacaa	gtcctggcgg	gggcataaga	25980
atgccatcat	ctcctgcttg	catgagtgcg	ggggcaacat	atccttcacg	cggcgctact	26040
tgtatttcca	ccatgggggtg	aactttccgc	gcaatgtttt	gcattactac	cgtcacctcc	26100
acagccccta	ctatagccag	caaatcccga	cagtctcgac	agataaagac	agcggcgggc	26160
acctccaaca	gaaaaccagc	agcggcagtt	agaaaaataca	caacaagtgc	agcaacagga	26220
ggattaaaga	ttacagccaa	cgagccagcg	caaaccggag	agttaagaaa	tcggatcttt	26280
ccaacctgtg	atgccatctt	ccagcagagt	cgggggtcaag	agcaggaact	gaaaataaaa	26340
aaccgatctc	tgcgttcgct	caccagaagt	tgtttgtatc	acaagagcga	agatcaactt	26400
cagcgactc	tcgaggacgc	cgaggctctc	ttcaacaagt	actgcgcgct	gactcttaaa	26460
gagtaggcag	cgaccgcgct	tattcaaaaa	aggcgggaat	tacatcatcc	tcgacatgag	26520
taaagaaatt	cccacgcctt	acatgtggag	ttatcaaccc	caaattgggt	tggcagcagg	26580
cgcctcccag	gactactcca	cccgcgatga	ttggctcagc	gccgggcctt	ctatgatttc	26640
tcgagttaat	gatatacgcg	cctaccgaaa	ccaaataactt	ttggaacagt	cagctcttac	26700
caccacgccc	cgccaacacc	ttaatcccag	aaattggccc	gccggccctag	tgtaccagga	26760
aagtcccgt	cccaccactg	tattacttcc	tcgagacgcc	caggccgaag	tccaaatgac	26820
taatgcaggt	gcgcagttag	ctggcggtc	caccctatgt	cgtcacaggc	ctcggcataa	26880
tataaaacgc	ctgatgatca	gaggccgagg	tatccagctc	aacgacgagt	cggtagctc	26940
tccgcttgg	ctacgaccag	acggaatctt	tcagattgcc	ggctgcggga	gatcttcctt	27000
caccctcgt	caggctgttc	tgactttgga	aagttcgtct	tcgcaacccc	gctcgggcgg	27060
aatcgggacc	gttcaatttg	tagaggagtt	tactccctct	gtctacttca	acccctctc	27120
cggatctcct	gggcactacc	cggacgagtt	cataccgaac	ttcgacgcga	ttagcgagtc	27180
agtggacggc	tacgattgat	gtctggtgac	cggctgagc	tatctcggct	gcgacatcta	27240
gaccactgcc	gccgctttcg	ctgctttgcc	cgggaactta	ttgagttcat	ctacttcgaa	27300
ctccccaagg	atcacctcca	aggctcggcc	cacggagtgc	ggattactat	cgaaggcaaa	27360
atagactctc	gcctgcaacg	aattttctcc	cagcgcccg	tgctgatcga	gcgagaccag	27420
ggaaacacca	cggtttccat	ctactgcatt	tgtaatcacc	ccggattgca	tgaagcctt	27480
tgtgtcttta	tgtgtactga	gtttaataaa	aactgaatta	agactctcct	acggactgcc	27540
gcttcttcaa	cccggtttt	acaaccagaa	gaacaaaact	tttctgtctg	tccaggactc	27600
tgttaaactt	acctttccta	ctcaciaaact	agaagctcaa	cgactacacc	gcttttccag	27660
aagcattttc	cctactaata	ctactttcaa	aaccggagggt	gagctccacg	gtctccctac	27720
agaaaaccct	tgggtggaag	cgggccttgt	agtactagga	attcttgccg	gtgggcttgt	27780
gattattctt	tgctacctat	acacaccttg	cttcactttc	ctagtgggtg	tgtggtattg	27840
gtttaaaaaa	tggggcccat	actagtcttg	cttgttttac	tttcgctttt	ggaaccgggt	27900

tctgccaat	acgatccatg	tctagacttt	gacccagaaa	actgcacact	tacttttgca	27960
cccgcacaa	gccgcatctg	tggagttctt	attaagtgcg	gatgggaatg	caggtccgtt	28020
gaaattacac	acaataacaa	aacctggaac	aataccttat	ccaccacatg	ggagccagga	28080
gttcccagag	ggtacactgt	ctctgtccga	ggtcctgacg	gttccatccg	cattagtaac	28140
aacactttca	ttttttctga	aatgtgcat	ctggccatgt	tcatgagcaa	acagtattct	28200
ctatggcctc	ctagcaagga	caacatcgta	acgtttctca	ttgcttattg	cttgtgcgct	28260
tgccttctta	ctgctttact	gtgcgtatgc	atacacctgc	ttgtaaccac	tcgcatcaaa	28320
aacgccaata	acaaagaaaa	aatgccttaa	cctctttctg	tttacagaca	tggcttctct	28380
tacatctctc	atatttgtca	gcattgtcac	tgccgctcac	ggacaaacag	tcgtctctat	28440
cccactagga	cataattaca	ctctcatagg	acccccaatc	acttcagagg	tcactctggac	28500
caaactggga	agcgttgatt	actttgatat	aatctgtaac	aaaacaaaac	caataatagt	28560
aacttgcaac	atacaaaatc	ttacattgat	taatgttagc	aaagtttaca	gcggttacta	28620
ttatggttat	gacagataca	gtagtcaata	tagaaattac	ttggttcgtg	ttaccagatt	28680
gaaaaccacg	aaaatgccaa	atatggcaaa	gattcgatcc	gatgacaatt	ctctagaaac	28740
ttttacatct	cccaccacac	ccgacgaaaa	aaacatccca	gattcaatga	ttgcaattgt	28800
tgcagcgggtg	gcagtgggtga	tggcactaat	aataatatgc	atgcttttat	atgcttgcg	28860
ctacaaaaag	tttcatccta	aaaaacaaga	tctcctacta	aggcttaaca	tttaatttct	28920
ttttatacag	ccatgggttc	cactaccaca	ttccttatgc	ttactagtct	cgcaactctg	28980
acttctgctc	gctcacacct	cactgtaact	ataggctcaa	actgcacact	aaaaggacct	29040
caaggtggtc	atgtcttttg	gtggagaata	tatgacaatg	gatggtttac	aaaaccatgt	29100
gaccaacctg	gtagattttt	ctgcaacggc	agagacctaa	ccattatcaa	cgtgacagca	29160
aatgacaaaag	gcttctatta	tggaaaccgac	tataaaaagta	gtttagatta	taacattatt	29220
gtactgccat	ctaccactcc	agcaccgccg	acaactactt	tctctagcag	cagtgtcgct	29280
aacaatacaa	tttccaatcc	aacctttgcc	gcgcttttaa	aacgcactgt	gaataattct	29340
acaacttcac	atacaacaat	ttccacttca	acaatcagca	tcactcgctgc	agtgacaatt	29400
ggaatactca	ttcttgtttt	taccataacc	tactacgcct	gctgctatag	aaaagacaaa	29460
cataaagggtg	atccattact	tagatttgat	atttaatttg	ttcttttttt	ttattttacag	29520
tatggtgaac	accaatcatg	gtacctagaa	atttcttctt	caccatactc	atctgtgctt	29580
ttaatgtttg	cgctactttc	acagcagtag	ccacagcaac	cccagactgt	ataggagcat	29640
ttgcttcccta	tgcacttttt	gcttttggtta	cttgcactctg	cgtatgtagc	atagtctgcc	29700
tggttatttaa	ttttttccaa	cttctagact	ggatccttgt	gcgaattgcc	tacctgcgcc	29760
accatccoga	ataccgcaac	caaaatatcg	cggcacttct	tagactcatc	taaaaccatg	29820
caggctatac	taccaatatt	tttgcttcta	ttgcttccct	acgctgtctc	aaccccgact	29880
gcctatagta	ctccaccaga	acaccttaga	aaatgcaaat	tccaacaacc	gtggctcattt	29940
cttgcttgct	atcgagaaaa	atcagaaatc	cccccaaatt	taataatgat	tgctggaata	30000
attaatataa	tctgttgcac	cataatttca	tttttgatat	accccttatt	tgattttggc	30060
tggaatgctc	ccaatgcaca	tgatcatcca	caagaccag	aggaacacat	ttccccacaa	30120
aacatgcaac	atccaatagc	gctaatagat	tacgaaagtg	aaccacaacc	cccactactc	30180
cctgctatta	gttacttcaa	cctaaccggc	ggagatgact	gaaacactca	ccacctccaa	30240
ttccgccgag	gatctgctcg	atatggacgg	ccgcgtctca	gaacaacgac	ttgcccactc	30300
acgcatccgc	cagcagcagg	aacgcgtggc	caaagagctc	agagatgtca	tccaaattca	30360
ccaatgcaaa	aaaggcatat	tctgtttggt	aaaacaagcc	aagatatcct	acgagatcac	30420
cgctactgac	catgcgctct	cttacgaact	tggcccccaa	cgacaaaaat	ttacctgcac	30480
ggtgggaatc	aaccccatag	ttatcaccca	acaaagtggg	gatactaagg	gttgcatcca	30540
ctgctcctgc	gattccatcg	agtgcacctc	cacctgtctg	aagaccctat	gcggcctaag	30600
agacctgcta	ccaatgaatt	aaaaaaaaat	gattaataaa	aaatcactta	cttgaaatca	30660
gcaataaggt	ctctgttgaa	attttctccc	agcagcacct	cacttccctc	ttcccaactc	30720
tggatttcta	aaccccgctc	agcggcatac	tttctccata	ctttaaaggg	gatgtcaaat	30780
tttagctcct	ctcctgtacc	cacaatcttc	atgtctttct	tcccagatga	ccaagagagt	30840
ccggctcagt	gactccttca	accctgtcta	cccctatgaa	gatgaaagca	cctcccaaca	30900
cccctttata	aaccaggggt	ttatttcccc	aaatggcttc	acacaaaagc	cagacggagt	30960
tcttacttta	aaatgtttta	ccccactaac	aaccacaggc	ggatctctac	agctaaaagt	31020
gggaggggga	cttacagtgg	atgacactga	tggtagctta	caagaaaaca	tacgtgctac	31080
agcaccattt	actaaaaata	atcactctgt	agaactatcc	attggaaatg	gattagaaac	31140
tcaaaaacaat	aaactatgtg	ccaaattggg	aaatgggtta	aaatttaaca	acggtgacat	31200
ttgtataaag	gatagtatta	acaccttatg	gactggaata	aacctccac	ctaactgtca	31260
aattgtggaa	aacactaata	caaatgatgg	caaacttact	ttagtattag	taaaaaatgg	31320

agggcttggt	aatggctacg	tgtctctagt	tgggtgtatca	gacactgtga	accaaagtgt	31380
cacacaaaag	acagcaaaca	tccaattaa	attatatattt	gactcttctg	gaaatctatt	31440
aactgaggaa	tcagacttaa	aaattccact	taaaaataaa	tcttctacag	cgaccagtga	31500
aactgtagcc	agcagcaaag	cctttatgcc	aagtactaca	gcttatccct	tcaacaccac	31560
tactagggat	agtgaaaact	acattcatgg	aatatgttac	tacatgacta	gttatgatag	31620
aagtctattt	cccttgaaca	tttctataat	gctaaacagc	cgtatgattt	cttccaatgt	31680
tgcctatgcc	atacaatttg	aatggaatct	aaatgcaagt	gaatctccag	aaagcaacat	31740
agctacgctg	accacatccc	cctttttctt	ttcttacatt	acagaagacg	acaactaaaa	31800
taaagttaa	gtgtttttat	ttaaaatcac	aaaattcgag	tagttatttt	gcctccacct	31860
tcccatttga	cagaatacac	caatctctcc	ccacgcacag	ctttaaacat	ttggatacca	31920
ttagagatag	acattgtttt	agattccaca	ttccaaacag	tttcagagcg	agccaatctg	31980
gggtcagtg	tagataaaaa	tccatcgcg	tagtctttta	aagcgctttc	acagtccaac	32040
tgtgcggat	gcgactccgg	agtttggatc	acggctcatct	ggaagaagaa	cgatgggaat	32100
cataatccga	aaacgggtatc	ggacgattgt	gtctcatcaa	acccacaagc	agccgctgtc	32160
tgcgtcgctc	cgtgcgactg	ctgtttatgg	gatcagggtc	cacagtttcc	tgaagcatga	32220
ttttaatagc	ccttaacatc	aactttctgg	tgcgatgcgc	gcagcaacgc	attctgattt	32280
cactcaaatc	tttgagtag	gtacaacaca	ttattacaat	attgtttaat	aaaccataat	32340
taaaagcgct	ccagccaaaa	ctcatatctg	atataatcgc	ccctgcatga	ccatcatacc	32400
aaagttaa	ataaattaaa	tgacgttccc	tcaaaaacac	actaccaca	tacatgatct	32460
cttttggcat	gtgcatatta	acaatctgtc	tgtaccatgg	acaacggttg	ttaatcatgc	32520
aaccaaatat	aaccttccgg	aaccacactg	ccaacaccgc	tccccagcc	atgcattgaa	32580
gtgaacctg	ctgattacaa	tgacaatgaa	gaaccaat	ctctcgaccg	tgaatcactt	32640
gagaatgaaa	aatatctata	gtggcacaa	atagacataa	atgcatgcat	cttctcataa	32700
ttttaactc	ctcaggattt	agaaacatat	cccagggaat	aggaagctct	tgacagaacag	32760
taaagctggc	agaacaagga	agaccacgaa	cacaacttac	actatgcata	gtcatagtat	32820
cacaatctgg	caacagcggg	tggctcttcag	tcatagaagc	tcggggttca	ttttcctcac	32880
aacgtggtaa	ctgggctctg	gtgtaagggt	gatgtctggc	gcatgatgtc	gagcgtgcgc	32940
gcaatggtag	cataatggag	ttgcttctctg	acattctcgt	attttgtata	gcaaaacgcg	33000
gacctggcag	aacacactct	tcttcgcctt	ctatcctgcc	gcttagcgtg	ttccgtgtga	33060
tagttcaagt	acagccacac	tcttaagtgg	gtcaaaaagaa	tgctggcttc	agttgtaatc	33120
aaaactccat	cgcactaat	tgttctgagg	aaatcatcca	cggtagcata	tgcaaatccc	33180
aaccaagcaa	tgaactgga	ttgcgtttca	agcaggagag	gagagggaag	agacggaaga	33240
accatgttaa	tttttattcc	aaacgatctc	gcagtacttc	aaattgtaga	tcgcgcagat	33300
ggcatctctc	gccccactg	tgttggtgaa	aaagcacagc	taaatcaaaa	gaaatgcgat	33360
tttcaagggtg	ctcaacgggtg	gcttccaaca	aagcctccac	gcgcacatcc	aagaacaaaa	33420
gaataccaaa	agaaggagca	ttttctaact	cctcaatcat	catattacat	tcctgcacca	33480
ttcccagata	attttcagct	ttccagcctt	gaattattcg	tgtcagttct	tggtgtaaat	33540
ccaatccaca	cattacaaac	aggtcccggg	gggcgccttc	caccaccatt	cttaaacaca	33600
ccctcataat	gacaaaatat	cttgctcctg	tgctcactgt	agcgaattga	gaaatggcaac	33660
atcaattgac	atgcccttgg	ctctaagttc	ttctttaagt	tctagttgta	aaaactctct	33720
catattatca	ccaaactgct	tagccagaag	ccccccggga	acaagagcag	gggacgctac	33780
agtgcagtag	aagcgcagac	ctccccaatt	ggctccagca	aaaacaagat	tggaataagc	33840
atattgggaa	ccaccagtaa	tatcatcgaa	gttgctggaa	atataatcag	gcagagtttc	33900
ttgtagaaat	tgaataaaaag	aaaaatttgc	caaaaaaaca	ttcaaaacct	ctgggatgca	33960
aatgcaatag	gttaccgcgc	tgcgctccaa	cattgttagt	tttgaattag	tctgcaaaaa	34020
taaaaaaaaa	acaagcgtca	tatcatagta	gcctgacgaa	caggtggata	aatcagctctt	34080
tccatcacia	gacaagccac	aggtctccca	gctcgaccct	cgtaaaacct	gtcatcgtga	34140
ttaaacaaca	gcaccgaaag	ttcctcgcg	tgaccagcat	gaataagtct	tgatgaagca	34200
tacaatccag	acatgttagc	atcagttaag	gagaaaaaac	agccaacata	gcctttgggt	34260
ataattatgc	ttaatcgtaa	gtatagcaaa	gccacccttc	gcggatacaa	agtaaaaggc	34320
acaggagaat	aaaaaatata	attatttctc	tgtgtgtgtt	taggcaacgt	cgcccccggt	34380
ccctctaaat	acacatacaa	agcctcatca	gccatggctt	accagagaaa	gtacagcggg	34440
cacacaaacc	acaagctcta	aagtcaactc	ccaacctstc	cacaatatat	atacacaagc	34500
cctaaactga	cgtaatggga	ctaaagtgt	aaaaatccc	ccaaacccaa	cacacacccc	34560
gaaactgcgt	caccaggga	aagtacagtt	tcaattccgc	aatcccaaca	agcgtcactt	34620
cctctttctc	acggtacgtc	acatcccatt	aacttacaac	gtcattttcc	cacggccgcg	34680
ccgccccctt	taaccgttaa	ccccacagcc	aatcaccaca	cggcccacac	tttttaaaat	34740

cacctcattt acatattggc accattccat ctataaggta tattattgat gatg

34794

<210> 45

<211> 180

<212> PRT

<213> adenoviridae

<220>

<221> SITE

<222> (1)..(180)

<223> /note="pCC536s E1B-21K sequence"

<400> 45

Met Glu Ala Trp Glu Cys Leu Glu Asp Phe Ser Ala Val Arg Asn Leu  
1 5 10 15

Leu Glu Gln Ser Ser Asn Ser Thr Ser Trp Phe Trp Arg Phe Leu Trp  
20 25 30

Gly Ser Ser Gln Ala Lys Leu Val Cys Arg Ile Lys Glu Asp Tyr Lys  
35 40 45

Trp Glu Phe Glu Glu Leu Leu Lys Ser Cys Gly Glu Leu Phe Asp Ser  
50 55 60

Leu Asn Leu Gly His Gln Ala Leu Phe Gln Glu Lys Val Ile Lys Thr  
65 70 75 80

Leu Asp Phe Ser Thr Pro Gly Arg Ala Ala Ala Val Ala Phe Leu  
85 90 95

Ser Phe Ile Lys Asp Lys Trp Ser Glu Glu Thr His Leu Ser Gly Gly  
100 105 110

Tyr Leu Leu Asp Phe Leu Ala Met His Leu Trp Arg Ala Val Val Arg  
115 120 125

His Lys Asn Arg Leu Leu Leu Leu Ser Ser Val Arg Pro Ala Ile Ile  
130 135 140

Pro Thr Glu Glu Gln Gln Gln Gln Glu Glu Ala Arg Arg Arg Arg  
145 150 155 160

Gln Glu Gln Ser Pro Trp Asn Pro Arg Ala Gly Leu Asp Pro Pro Val  
165 170 175

Glu Glu Ala Glu  
180

<210> 46

<211> 176

<212> PRT

<213> adenoviridae

<220>

<221> SITE  
 <222> (1)..(176)  
 <223> /note="Ad5. E1B-21K sequence"

<400> 46

```

Met Glu Ala Trp Glu Cys Leu Glu Asp Phe Ser Ala Val Arg Asn Leu
 1             5             10             15

Leu Glu Gln Ser Ser Asn Ser Thr Ser Trp Phe Trp Arg Phe Leu Trp
             20             25             30

Gly Ser Ser Gln Ala Lys Leu Val Cys Arg Ile Lys Glu Asp Tyr Lys
             35             40             45

Trp Glu Phe Glu Glu Leu Leu Lys Ser Cys Gly Glu Leu Phe Asp Ser
 50             55             60

Leu Asn Leu Gly His Gln Ala Leu Phe Gln Glu Lys Val Ile Lys Thr
 65             70             75             80

Leu Asp Phe Ser Thr Pro Gly Arg Ala Ala Ala Ala Val Ala Phe Leu
             85             90             95

Ser Phe Ile Lys Asp Lys Trp Ser Glu Glu Thr His Leu Ser Gly Gly
             100            105            110

Tyr Leu Leu Asp Phe Leu Ala Met His Leu Trp Arg Ala Val Val Arg
            115            120            125

His Lys Asn Arg Leu Leu Leu Leu Ser Ser Val Arg Pro Ala Ile Ile
            130            135            140

Pro Thr Glu Glu Gln Gln Gln Gln Gln Glu Glu Ala Arg Arg Arg Arg
            145            150            155            160

Gln Glu Gln Ser Pro Trp Asn Pro Arg Ala Gly Leu Asp Pro Arg Glu
            165            170            175

```

<210> 47  
 <211> 180  
 <212> PRT  
 <213> adenoviridae

<220>  
 <221> SITE  
 <222> (1)..(180)  
 <223> /note="Ad35.E1B-21K sequence"

<400> 47

```

Met Glu Val Trp Ala Ile Leu Glu Asp Leu Arg Lys Thr Arg Gln Leu
 1             5             10             15

Leu Glu Ser Ala Ser Asp Gly Val Ser Gly Phe Trp Arg Phe Trp Phe
            20             25             30

Ala Ser Glu Leu Ala Arg Val Val Phe Arg Ile Lys Gln Asp Tyr Lys

```

35                      40                      45  
 Gln Glu Phe Glu Lys Leu Leu Val Asp Cys Pro Gly Leu Phe Glu Ala  
     50                      55                      60  
 Leu Asn Leu Gly His Gln Val His Phe Lys Glu Lys Val Leu Ser Val  
     65                      70                      75                      80  
 Leu Asp Phe Ser Thr Pro Gly Arg Thr Ala Ala Val Ala Phe Leu  
                     85                      90                      95  
 Thr Phe Ile Leu Asp Lys Trp Ile Pro Gln Thr His Phe Ser Arg Gly  
                     100                      105                      110  
 Tyr Val Leu Asp Phe Ile Ala Thr Ala Leu Trp Arg Thr Trp Lys Val  
                     115                      120                      125  
 Arg Lys Met Arg Thr Ile Leu Gly Tyr Trp Pro Val Gln Pro Leu Gly  
                     130                      135                      140  
 Val Ala Gly Ile Leu Arg His Pro Pro Val Met Pro Ala Val Leu Glu  
     145                      150                      155                      160  
 Glu Glu Gln Gln Glu Asp Asn Pro Arg Ala Gly Leu Asp Pro Pro Val  
                     165                      170                      175  
 Glu Glu Ala Glu  
                     180

<210> 48  
 <211> 494  
 <212> PRT  
 <213> adenoviridae

<220>  
 <221> SITE  
 <222> (1)..(494)  
 <223> /note="pCC536s E1B-55K sequence"

<400> 48  
 Met Glu Arg Arg Asn Pro Ser Glu Arg Gly Val Pro Ala Gly Phe Ser  
     1                      5                      10                      15  
 Gly His Ala Ser Val Glu Ser Gly Cys Glu Thr Gln Glu Ser Pro Ala  
                     20                      25                      30  
 Thr Val Val Phe Arg Pro Pro Gly Asp Asn Thr Asp Gly Gly Ala Ala  
                     35                      40                      45  
 Ala Ala Ala Gly Gly Ser Gln Ala Ala Ala Ala Gly Ala Glu Pro Met  
                     50                      55                      60  
 Glu Pro Glu Ser Arg Pro Gly Pro Ser Ser Gly Gly Gly Gly Val Ala  
     65                      70                      75                      80  
 Asp Leu Ser Pro Glu Leu Gln Arg Val Leu Thr Gly Ser Thr Ser Thr



85

90

95

Gly Arg Asp Arg Gly Val Lys Arg Glu Arg Ala Ser Ser Gly Thr Asp  
 100 105 110

Ala Arg Ser Glu Leu Ala Leu Ser Leu Met Ser Arg Arg Arg Pro Glu  
 115 120 125

Thr Ile Trp Trp His Glu Val Gln Lys Glu Gly Arg Asp Glu Val Ser  
 130 135 140

Val Leu Gln Glu Lys Tyr Ser Leu Glu Gln Val Lys Thr Cys Trp Leu  
 145 150 155 160

Glu Pro Glu Asp Asp Trp Ala Val Ala Ile Lys Asn Tyr Ala Lys Ile  
 165 170 175

Ala Leu Arg Pro Asp Lys Gln Tyr Lys Ile Ser Arg Arg Ile Asn Ile  
 180 185 190

Arg Asn Ala Cys Tyr Ile Ser Gly Asn Gly Ala Glu Val Val Ile Asp  
 195 200 205

Thr Gln Asp Lys Thr Val Ile Arg Cys Cys Met Met Asp Met Trp Pro  
 210 215 220

Gly Val Val Gly Met Glu Ala Val Thr Phe Val Asn Val Lys Phe Arg  
 225 230 235 240

Gly Asp Gly Tyr Asn Gly Ile Val Phe Met Ala Asn Thr Lys Leu Ile  
 245 250 255

Leu His Gly Cys Ser Phe Phe Gly Phe Asn Asn Thr Cys Val Asp Ala  
 260 265 270

Trp Gly Gln Val Ser Val Arg Gly Cys Ser Phe Tyr Ala Cys Trp Ile  
 275 280 285

Ala Thr Ala Gly Arg Thr Lys Ser Gln Leu Ser Leu Lys Lys Cys Ile  
 290 295 300

Phe Gln Arg Cys Asn Leu Gly Ile Leu Asn Glu Gly Glu Ala Arg Val  
 305 310 315 320

Arg His Cys Ala Ser Thr Asp Thr Gly Cys Phe Ile Leu Ile Lys Gly  
 325 330 335

Asn Ala Ser Val Lys His Asn Met Ile Cys Gly Ala Ser Asp Glu Arg  
 340 345 350

Pro Tyr Gln Met Leu Thr Cys Ala Gly Gly His Cys Asn Met Leu Ala  
 355 360 365

Thr Val His Ile Val Ser His Gln Arg Lys Lys Trp Pro Val Phe Asp  
 370 375 380

His Asn Val Leu Thr Lys Cys Thr Met His Ala Gly Gly Arg Arg Gly

385                      390                      395                      400  
 Met Phe Met Pro Tyr Gln Cys Asn Met Asn His Val Lys Val Leu Leu  
                                  405                      410                      415  
 Glu Pro Asp Ala Phe Ser Arg Met Ser Leu Thr Gly Ile Phe Asp Met  
                                  420                      425                      430  
 Asn Thr Gln Ile Trp Lys Ile Leu Arg Tyr Asp Asp Thr Arg Ser Arg  
                                  435                      440                      445  
 Val Arg Ala Cys Glu Cys Gly Gly Lys His Ala Arg Phe Gln Pro Val  
                                  450                      455                      460  
 Cys Val Asp Val Thr Glu Asp Leu Arg Pro Asp His Leu Val Ile Ala  
 465                                   470                      475                      480  
 Arg Thr Gly Ala Glu Phe Gly Ser Ser Gly Glu Glu Thr Asp  
                                  485                      490

<210> 49  
 <211> 494  
 <212> PRT  
 <213> adenoviridae  
  
 <220>  
 <221> SITE  
 <222> (1)..(494)  
 <223> /note="Ad35. E1B-55K sequence"

<400> 49  
 Met Asp Pro Ala Asp Ser Phe Gln Gln Gly Ile Arg Phe Gly Phe His  
   1                                  5                                  10                                  15  
 Ser His Ser Ile Val Glu Asn Met Glu Gly Ser Gln Asp Glu Asp Asn  
                                   20                                  25                                  30  
 Leu Arg Leu Leu Ala Ser Ala Ala Phe Gly Cys Ser Gly Asn Pro Glu  
                                   35                                  40                                  45  
 Ala Ser Thr Gly His Ala Ser Gly Ser Gly Gly Gly Thr Ala Arg Gly  
                                   50                                  55                                  60  
 Gln Pro Glu Ser Arg Pro Gly Pro Ser Ser Gly Gly Gly Gly Val Ala  
 65                                  70                                  75                                  80  
 Asp Leu Ser Pro Glu Leu Gln Arg Val Leu Thr Gly Ser Thr Ser Thr  
                                   85                                  90                                  95  
 Gly Arg Asp Arg Gly Val Lys Arg Glu Arg Ala Ser Ser Gly Thr Asp  
                                   100                                  105                                  110  
 Ala Arg Ser Glu Leu Ala Leu Ser Leu Met Ser Arg Arg Arg Pro Glu  
                                   115                                  120                                  125  
 Thr Ile Trp Trp His Glu Val Gln Lys Glu Gly Arg Asp Glu Val Ser

130	135	140
Val Leu Gln Glu Lys Tyr Ser Leu Glu Gln Val Lys Thr Cys Trp Leu 145 150 155 160		
Glu Pro Glu Asp Asp Trp Ala Val Ala Ile Lys Asn Tyr Ala Lys Ile 165 170 175		
Ala Leu Arg Pro Asp Lys Gln Tyr Lys Ile Ser Arg Arg Ile Asn Ile 180 185 190		
Arg Asn Ala Cys Tyr Ile Ser Gly Asn Gly Ala Glu Val Val Ile Asp 195 200 205		
Thr Gln Asp Lys Thr Val Ile Arg Cys Cys Met Met Asp Met Trp Pro 210 215 220		
Gly Val Val Gly Met Glu Ala Val Thr Phe Val Asn Val Lys Phe Arg 225 230 235 240		
Gly Asp Gly Tyr Asn Gly Ile Val Phe Met Ala Asn Thr Lys Leu Ile 245 250 255		
Leu His Gly Cys Ser Phe Phe Gly Phe Asn Asn Thr Cys Val Asp Ala 260 265 270		
Trp Gly Gln Val Ser Val Arg Gly Cys Ser Phe Tyr Ala Cys Trp Ile 275 280 285		
Ala Thr Ala Gly Arg Thr Lys Ser Gln Leu Ser Leu Lys Lys Cys Ile 290 295 300		
Phe Gln Arg Cys Asn Leu Gly Ile Leu Asn Glu Gly Glu Ala Arg Val 305 310 315 320		
Arg His Cys Ala Ser Thr Asp Thr Gly Cys Phe Ile Leu Ile Lys Gly 325 330 335		
Asn Ala Ser Val Lys His Asn Met Ile Cys Gly Ala Ser Asp Glu Arg 340 345 350		
Pro Tyr Gln Met Leu Thr Cys Ala Gly Gly His Cys Asn Met Leu Ala 355 360 365		
Thr Val His Ile Val Ser His Gln Arg Lys Lys Trp Pro Val Phe Asp 370 375 380		
His Asn Val Leu Thr Lys Cys Thr Met His Ala Gly Gly Arg Arg Gly 385 390 395 400		
Met Phe Met Pro Tyr Gln Cys Asn Met Asn His Val Lys Val Leu Leu 405 410 415		
Glu Pro Asp Ala Phe Ser Arg Met Ser Leu Thr Gly Ile Phe Asp Met 420 425 430		
Asn Thr Gln Ile Trp Lys Ile Leu Arg Tyr Asp Asp Thr Arg Ser Arg		

435                      440                      445  
 Val Arg Ala Cys Glu Cys Gly Gly Lys His Ala Arg Phe Gln Pro Val  
     450                      455                      460  
 Cys Val Asp Val Thr Glu Asp Leu Arg Pro Asp His Leu Val Ile Ala  
     465                      470                      475                      480  
 Arg Thr Gly Ala Glu Phe Gly Ser Ser Gly Glu Glu Thr Asp  
                     485                      490  
  
 <210> 50  
 <211> 496  
 <212> PRT  
 <213> adenoviridae  
  
 <220>  
 <221> SITE  
 <222> (1)..(496)  
 <223> /note="Ad5. E1B-55K sequence"  
  
 <400> 50  
 Met Glu Arg Arg Asn Pro Ser Glu Arg Gly Val Pro Ala Gly Phe Ser  
     1                      5                      10                      15  
 Gly His Ala Ser Val Glu Ser Gly Cys Glu Thr Gln Glu Ser Pro Ala  
                     20                      25                      30  
 Thr Val Val Phe Arg Pro Pro Gly Asp Asn Thr Asp Gly Gly Ala Ala  
                     35                      40                      45  
 Ala Ala Ala Gly Gly Ser Gln Ala Ala Ala Ala Gly Ala Glu Pro Met  
     50                      55                      60  
 Glu Pro Glu Ser Arg Pro Gly Pro Ser Gly Met Asn Val Val Gln Val  
     65                      70                      75                      80  
 Ala Glu Leu Tyr Pro Glu Leu Arg Arg Ile Leu Thr Ile Thr Glu Asp  
                     85                      90                      95  
 Gly Gln Gly Leu Lys Gly Val Lys Arg Glu Arg Gly Ala Cys Glu Ala  
                     100                      105                      110  
 Thr Glu Glu Ala Arg Asn Leu Ala Phe Ser Leu Met Thr Arg His Arg  
                     115                      120                      125  
 Pro Glu Cys Ile Thr Phe Gln Gln Ile Lys Asp Asn Cys Ala Asn Glu  
     130                      135                      140  
 Leu Asp Leu Leu Ala Gln Lys Tyr Ser Ile Glu Gln Leu Thr Thr Tyr  
     145                      150                      155                      160  
 Trp Leu Gln Pro Gly Asp Asp Phe Glu Glu Ala Ile Arg Val Tyr Ala  
                     165                      170                      175  
 Lys Val Ala Leu Arg Pro Asp Cys Lys Tyr Lys Ile Ser Lys Leu Val

180										185					190				
Asn	Ile	Arg	Asn	Cys	Cys	Tyr	Ile	Ser	Gly	Asn	Gly	Ala	Glu	Val	Glu				
		195						200					205						
Ile	Asp	Thr	Glu	Asp	Arg	Val	Ala	Phe	Arg	Cys	Ser	Met	Ile	Asn	Met				
		210				215						220							
Trp	Pro	Gly	Val	Leu	Gly	Met	Asp	Gly	Val	Val	Ile	Met	Asn	Val	Arg				
		225				230					235				240				
Phe	Thr	Gly	Pro	Asn	Phe	Ser	Gly	Thr	Val	Phe	Leu	Ala	Asn	Thr	Asn				
				245					250					255					
Leu	Ile	Leu	His	Gly	Val	Ser	Phe	Tyr	Gly	Phe	Asn	Asn	Thr	Cys	Val				
			260					265						270					
Glu	Ala	Trp	Thr	Asp	Val	Arg	Val	Arg	Gly	Cys	Ala	Phe	Tyr	Cys	Cys				
		275					280						285						
Trp	Lys	Gly	Val	Val	Cys	Arg	Pro	Lys	Ser	Arg	Ala	Ser	Ile	Lys	Lys				
		290				295					300								
Cys	Leu	Phe	Glu	Arg	Cys	Thr	Leu	Gly	Ile	Leu	Ser	Glu	Gly	Asn	Ser				
		305			310					315					320				
Arg	Val	Arg	His	Asn	Val	Ala	Ser	Asp	Cys	Gly	Cys	Phe	Met	Leu	Val				
				325					330					335					
Lys	Ser	Val	Ala	Val	Ile	Lys	His	Asn	Met	Val	Cys	Gly	Asn	Cys	Glu				
			340					345					350						
Asp	Arg	Ala	Ser	Gln	Met	Leu	Thr	Cys	Ser	Asp	Gly	Asn	Cys	His	Leu				
		355					360					365							
Leu	Lys	Thr	Ile	His	Val	Ala	Ser	His	Ser	Arg	Lys	Ala	Trp	Pro	Val				
		370				375					380								
Phe	Glu	His	Asn	Ile	Leu	Thr	Arg	Cys	Ser	Leu	His	Leu	Gly	Asn	Arg				
		385			390					395					400				
Arg	Gly	Val	Phe	Leu	Pro	Tyr	Gln	Cys	Asn	Leu	Ser	His	Thr	Lys	Ile				
				405					410					415					
Leu	Leu	Glu	Pro	Glu	Ser	Met	Ser	Lys	Val	Asn	Leu	Asn	Gly	Val	Phe				
			420					425					430						
Asp	Met	Thr	Met	Lys	Ile	Trp	Lys	Val	Leu	Arg	Tyr	Asp	Glu	Thr	Arg				
		435					440					445							
Thr	Arg	Cys	Arg	Pro	Cys	Glu	Cys	Gly	Gly	Lys	His	Ile	Arg	Asn	Gln				
		450				455					460								
Pro	Val	Met	Leu	Asp	Val	Thr	Glu	Glu	Leu	Arg	Pro	Asp	His	Leu	Val				
		465			470				475					480					
Leu	Ala	Cys	Thr	Arg	Ala	Glu	Phe	Gly	Ser	Ser	Asp	Glu	Asp	Thr	Asp				

485

490

495